MiR-30b-5p functions as a tumor suppressor in cell proliferation, metastasis and epithelial-to-mesenchymal transition by targeting G-protein subunit α-13 in renal cell carcinoma

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ARTICLE INFO

Keywords:
MiR-30b-5p
Proliferation
Metastasis
G-protein subunit α-13 (GNA13)
Renal cell carcinoma (RCC)

ABSTRACT

Increasing evidence has demonstrated that aberrant microRNAs (miRNAs) play important roles in the pathogenesis of most human malignancies. The purpose of this study was to explore the role of miR-30b-5p in human RCC. In the current study, we firstly found that the expression levels of miR-30b-5p were lower in both RCC tissues and cell lines. Then, we found that enforced miR-30b-5p expression and knockdown of GNA13 significantly suppressed the proliferation, invasion, migration and EMT of RCC cell lines. In addition, miR-30b-5p directly targeted GNA13 and repressed its expression. Furthermore, re-expression of GNA13 (without the 3′-UTR) could partially abrogate the miR-30b-5p-induced cell proliferation and metastasis inhibition. Taken together, these findings indicated that miR-30b-5p acts as a novel tumor suppressor to regulate RCC cell proliferation, metastasis and EMT through downregulation of GNA13 expression. Therefore, miR-30b-5p may be considered a potential biomarker for the diagnosis of RCC.

1. Introduction

Renal cell carcinoma (RCC) represents the leading cause of cancer death among urological malignancies. Apart from surgical therapies, current targeted therapies have improved survival in patients with advanced disease but complete response occurs rarely (Haddad and Margulis, 2015). Therefore, analysis of the molecular mechanisms underlying RCC development and progression and studies of novel oncogenic pathways based on current genome-based approaches could significantly improve diagnosis, therapy, and prevention of the disease.

MicroRNAs (miRNAs) inhibit gene expression at the posttranscriptional level by blocking mRNA translation or degrading target mRNAs (de Planell-Saguer and Rodicio, 2011). Aberrant levels of the miRNAs have been found in a variety of cancers, and could modulate tumor development, differentiation, proliferation, invasion, and metastasis (Kasinski and Slack, 2011). Furthermore, miRNAs are involved in the pathogenesis of tumors acting as tumor suppressors or oncogenes (Ruﬁno-Palomares et al., 2014). Thus, the expression profiles of miRNAs can be conductive to the diagnosis, prognosis and therapies of cancers (Garzon and Marcucci, 2012; Cho, 2010). MicroRNA-30b (miR-30b), a member of miR-30 family, has been reported to be involved in the development of various human malignancies. Early studies have shown that miR-30b expression is particularly increased and impairs TRAIL-dependent apoptosis by targeting caspase-3 in TRAIL-resistant glioma cells (Quintavalle et al., 2013). A previous study also showed that high miR-30b expression in combination with other miRNAs significantly predicts a shorter recurrence-free survival in hepatocellular carcinoma (Huang et al., 2012). Another recent study reported that upregulation of miR-30b plays a role in blocking of terminal B cell differentiation in primary central nervous system tumor.
lymphoma (PCNSL) (Fischer et al., 2011). Nevertheless, the role of miR-30b-5p in RCC remains to be elucidated.

A prominent molecular event in the early stages of distant metastasis is the epithelial-to-mesenchymal transition (EMT), a complex process characterized by enhanced cell migration and invasion (Mani et al., 2008). EMT is characterized by the loss of epithelial cell markers such as E-cadherin and gain of mesenchymal phenotypes with expression of mesenchymal proteins including N-cadherin and vimentin (Yang and Weinberg, 2008). A growing body of evidence from clinical and experimental studies has described the role of EMT in RCC dissemination and the requirement of MET in efficient metastasis (Brabletz et al., 2005).

The purpose of this study was to investigate the effect and mechanisms of miR-30b-5p in cell proliferation, metastatic and EMT in RCC. The present findings may give us insight into how miR-30b-5p acts as a tumor suppressor in RCC cells and suggest a novel biomarker or therapeutic strategy for treatment of RCC.

2. Materials and methods

2.1. Human tissue samples

Matched fresh renal carcinoma specimens and adjacent non-tumorous tissue specimens were obtained from 16 patients (5 in clinical stage I, 4 in clinical stage II, 3 in clinical stage IV, 8 in TNM stage I, 5 in TNM stage II, 3 in TNM stage III) at the Department of Urology, YanTai Yuhuangding Hospital, Yantai, China, from 2010 to 2014. The specimens were immediately frozen in liquid nitrogen and then stored at −80°C before RNA isolation. All renal cell carcinoma specimens were confirmed by pathological examination. Prior written informed consent to participate was obtained, and the study protocol was approved by the Medical Ethics Committee of the Affiliated Hospital of Yantai Yuhuangding before using the clinical specimens (Certificate no.PJ2012130).

2.2. Cell culture

The human RCC cell lines (786-O, Caki-1, Caki-2, and ACHN), and normal renal proximal tubular cells (HK-2) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). 786-O were maintained in RPMI-1640 (Gibco, USA), Caki-1 and Caki-2 were maintained in McCoy’s 5A medium (Gibco, USA), HK-2 and ACHN cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco, USA). All these mediums were supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C.

2.3. RNA isolation and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Reverse transcription of microRNA and mRNA were done using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) and miProfile™ miRNA qPCR Primer (GeneCopoeia, Guangzhou, China). RT-qPCR analysis of miRNA was performed with the Platinum SYBR Green qPCR Supermix UDG kit (Invitrogen, Carlsbad, CA) using synthesized primers from GeneCopoeia (Guangzhou, China). U6 or GAPDH levels were used as internal controls, and fold changes were calculated using the 2^(-ΔΔCt) method. Each experiment was performed in triplicate.

2.4. Western blot

Proteins were extracted by nuclear-cytosol extraction kit (Applygen, China) according to the instructions. Protein concentration was measured using the Bicinchoninic acid (BCA) protein assay kit (Pierce,
USA). For western blot analysis, equal amounts of total protein were boiled and separated by SDS-PAGE. After electrophoresis, protein were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk in phosphate-buffered saline (PBS) at room temperature for 1 h, and then incubated overnight at 4 °C with the following primary antibodies: Monoclonal mouse anti-human fibronectin, polyclonal rabbit anti-human N-cadherin, polyclonal rabbit anti-human vimentin, polyclonal rabbit anti-human E-cadherin (Santa Cruz, CA, USA) or with monoclonal rabbit anti-human GAPDH (Abcam, Cambridge, MA, USA). The membranes were washed three times with TBST, and then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz, USA) for 1 h at room temperature. Blots were processed using an enhanced chemiluminescence kit (Santa Cruz Biotechnology Inc.) and exposed to film (Kodak, Rochester, NY, USA). The expression levels of the target proteins were normalized to those of GAPDH.

2.5. Cell proliferation assay

The cells were transfected with miR-30b-5p mimics or negative control to investigate the impact of miR-30b-5p on cell proliferation of RCC cells. 48 h after transfection, cells were seeded into 96-well plates at a density of 2 × 10^3 cells/well and cultured for 24, 48, 72, or 96 h. Cell proliferation was determined using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies) in accordance with the manufacturer's protocol. Absorbance was detected at the wavelength of 450 nm. Three wells were measured for cell viability in each treatment group.

2.6. Transwell invasion assay

To determine cell invasion, Matrigel-coated invasion chambers (Invitrogen) were used according to the manufacturer's protocol. The cells transfected with miR-30b-5p mimics or negative control were suspended in 200 μl serum-free RPMI-1640 medium and seeded on the upper chamber. The lower chamber was filled with 10% FBS as the chemoattractant. After 48 h, cells on the upper side of the membrane were wiped off, and cells on the lower side of the membrane were fixed and stained with crystal violet solution. The cells under the microscopic fields in each chamber were photographed and counted, and the values were expressed as fold induction. All invasion assays were performed in triplicate in at least three independent experiments.
2.7. Scratch migration assay

To determine cell migration, the cells transfected with miR-30b-5p mimics or negative control were seeded into 12-well plates and cultured overnight. Before scratching, the cells were starved for 24 h in medium with 1% FBS. Similar sized wounds were introduced to monolayer cells using a sterile white pipette tip. Wounded monolayer cells were washed three times by PBS to remove cell debris and then cultured. The speed of wound closure was monitored and photographed at 48 h. All of the experiments were performed in triplicate.

2.8. Plasmid vectors and siRNA transfection

To overexpression of miR-30b-5p, the miR-30b-5p mimics (miR-30b-5p p) and its negative control RNA oligonucleotides (NC) were transfected into both 768-O and ACHN cells using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). After transfection for 48 h, the transfection medium was swapped with normal medium, and the cells were harvested to measure miR-30b-5p transfection efficiency or used in the subsequent experiments.

GNA13 siRNA (siGNA13; TACGTCCAAGGTCGGGCAGGAAGA) and its negative control siRNA (simock) were transfected to knockdown of GNA13 in 768-O and ACHN cells according to the instruction of Lipofectamine 2000 (Invitrogen). The cells were harvested to measure miR-30b-5p transfection efficiency or used in the subsequent experiments after transfection for 48 h.

2.9. Luciferase activity assay

Luciferase activity assays were performed using the Dual-Luciferase reporter assay System (Promega; Madison, AL, USA). Cells were seeded in triplicate in 24-well plates one day before transfection. Wt or Mut 3′-UTR vectors and the control vector pGL3 (Promega) coding for Renilla luciferase were co-transfected with miR-30b-5p mimics or negative control into the cells using Lipofectamine 2000 reagent (Invitrogen; Grand Island, NY, USA) according to manufacturer’s protocol. After 48 h, cells were harvested and lysed, and the luciferase activity was assayed using the Dual-Luciferase Assay Reporter System (Promega). The firefly luciferase fluorescence was normalized to Renilla, and the relative ratios of firefly to Renilla activity were reported.

2.10. Statistical analyses

All of the statistical calculations were performed using the SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± SD from at least three independent experiments. Comparisons were made using one-way analysis of variance, and Student’s t-test was used to analyze the differences between two experimental groups. *P < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. miR-30b-5p was down-regulated in RCC tissues and cell lines and was associated with metastasis

To investigate the potential significance of miR-30b-5p in development and progression of renal carcinoma, we first examined the expression levels of miR-30b-5p in RCC tissues and cell lines using qRT-PCR. MiR-30b-5p was significantly lower in RCC tissues than in adjacent normal tissues (Fig.1A). We further assessed the expression level of miR-30b-5p in RCC cell lines. In parallel, as shown in Fig. 1B, miR-30b-5p expression was found to be markedly decreased in RCC cell lines than that in normal renal cell line HK-2. These data suggested that miR-30b-5p expression was downregulated in RCC. In addition, to determine the relationship between metastasis and miR-30b-5p expression, we examined data regarding the clinical stage and TNM stage of the patients with RCC involved in the study. The data showed decreased expression of miR-30b-5p in tumors with higher grade and higher TNM stage (Fig.1C and D), suggesting that lower miR-30b-5p expression is associated with more aggressive cancer.

3.2. MiR-30b-5p suppressed proliferation and metastasis of RCC in vitro

To investigate the potential function of miR-30b-5p in RCC tumorigenesis, we first determined the effects of miR-30b-5p overexpression on cell proliferation using CCK-8 assay. Successful overexpression of miR-30b-5p in the RCC cell lines 786-O and ACHN were confirmed by qRT-PCR (Fig.2A). We found that overexpression of miR-30b-5p significantly decreased the proliferation in the two RCC cell lines (Fig.2D). We also performed Transwell invasion assay and scratch...
migration assay to investigate the effect of miR-30b-5p on RCC metastasis. The results as shown in Fig. 2B, Transwell invasion assays revealed that miR-30b-5p overexpression could markedly suppressed the invasive abilities of RCC cell lines. The scratch migration assay demonstrated that ectopic miR-30b-5p reduced the migration capacity of both cell lines (Fig.2C), consistent with the results of the cell invasion assay. Taken together, these results suggested that highly expressed miR-30b-5p suppressed the proliferation and metastasis of RCC cells.

3.3. MiR-30b-5p regulated epithelial-mesenchymal transition (EMT) in RCC cells

EMT is a key process in cancer metastasis. Migration and invasion assays reveal lower migratory and invasive abilities in the cell in which miR-30b-5p expression was also lower than the NC. Western blotting was performed to determine EMT marker protein expression levels (α-catenin, E-cadherin, vimentin and N-cadherin) in 786-O and ACHN cells. The results showed that epithelial markers (α-catenin and E-cadherin) were upregulated, while mesenchymal markers (vimentin and N-cadherin) were downregulated by restoring miR-30b-5p expression by means of stable transfection (Fig.2E). These data suggested that miR-30b-5p can suppress EMT.

3.4. MiR-30b-5p directly targeted GNA13

To elucidate the underlying mechanisms by which miR-30b-5p exerts its function, we explored targets of miR-30b-5p using the TargetScan bioinformatics algorithm. Our analysis revealed that GNA13 was a potential target of miR-30b-5p based on putative target sequences at the GNA13 3′-UTR (Fig. 3A). To confirm GNA13 as a direct target of miR-30b-5p, we engineered luciferase reporter constructs containing the wild-type (WT) or mutant (Mut) 3′-UTR of the GNA13 gene. Luciferase reporter assays showed that miR-30b-5p significantly decreased the luciferase activity of the GNA13 3′-UTR but not that of the mutant in the 786-O and ACHN cells (Fig. 3B and C). qRT-PCR and western blot analyses showed that overexpression of miR-30b-5p...
significantly downregulated the expression of GNA13 at the mRNA and protein levels in both RCC cell lines (Fig. 3D–F). These data provide compelling evidence that GNA13 was direct targets of miR-30b-5p, and that its expression was negatively regulated by miR-30b-5p in RCC cells.

3.5. GNA13 silencing recapitulated the effects of miR-30b-5p in RCC

We examined the mRNA or protein expression of GNA13 in 786-O and ACHN cells. The data revealed that the expression level of GNA13 were markedly upregulated in both 786-O and ACHN cells compared to the normal renal cell line HK-2 (Fig. 4A and B). To explore the function of GNA13 in RCC, 786-O cells were transfected with siGNA13, and the silencing efficiency of GNA13 in the cells was detected by qRT-PCR and Western blot (Fig. 4C and D). Next, we investigated whether GNA13 is instrumental to the proliferation and metastasis of RCC cells. The CCK-8 assay revealed that knockdown of GNA13 also inhibited RCC proliferation (Fig. 4E). Furthermore, Transwell invasion assay and Scratch migration assay suggested that downregulation of GNA13 inhibited the invasion and migration capability of RCC (Fig. 4F and G). GNA13 silencing induced a very similar phenotype to miR-30b-5p expression in RCC. These results suggested that miR-30b-5p downregulated GNA13, thus suppressing RCC proliferation and metastasis.

3.6. Reintroduction of GNA13 rescued the suppressive effect of miR-30b-5p on the proliferation and metastasis of RCC cells

If GNA13 indeed serves as a direct functional target of miR-30b-5p, reintroduction of GNA13 into miR-30b-5p-expressing cells should antagonize the effects of miR-30b-5p. To address this hypothesis, we co-transfected miR-30b-5p mimics along with GNA13 into 786-O cells. We found that GNA13 overexpression abrogated the suppressive effect of miR-30b-5p on the proliferation (Fig. 4H), invasion (Fig. 4I) and migration (Fig. 4J) of RCC cells. These findings demonstrated that GNA13 reintroduction abrogated miR-30b-5p-induced cellular behaviors, suggesting that GNA13 is a functional mediator of miR-30b-5p in RCC.

4. Discussion

Recently, large numbers of evidence has indicated that miRNAs are frequently in a variety of human malignancies (Yates et al., 2013). Our current study showed that miR-30b-5p functions as a tumor suppressor in RCC, and GNA13 was a functional target of miR-30b-5p. We demonstrated that miR-30b-5p was markedly downregulated in RCC tissues and cell lines. Furthermore, we found that overexpression of miR-30b-5p in RCC resulted in inhibition of GNA13 expression, and suppressed proliferation, metastasis and EMT of RCC. We demonstrated that miR-30b-5p acts as a novel growth and metastasis suppressor by targeting GNA13 in RCC.

Studies showed a direct link between miRNA function and oncogenesis which is supported by examining the expression of miRNAs in clinical samples (Dassow and Aigner, 2013). Therefore, we investigated the expression of miR-30b-5p in RCC tissues and cell lines. We found that miR-30b-5p was significantly downregulated in RCC tissues relative to adjacent nontumor tissues, which is consistent with the investigation of Ge et al. (Ge et al., 2015) who indicated that miR-30b was downregulated in RCC tissues and might be serve as an independent prognostic factor in RCC. Meanwhile, we tested the miR-30b-5p levels in RCC cell lines and found that there was less miR-30b-5p in RCC cells than that in normal renal cells. These findings suggest that miR-30b-5p may be a potential biomarker for RCC. A considerable amount of evidence indicates that aberrant miRNAs can either suppress or promote the proliferation, apoptosis, and metastasis of RCC (Catto et al., 2011). More important, in this study, we reviewed the role of miR-30b-5p in the oncogenesis and metastasis of RCC, and we found that upregulated expression of miR-30b-5p could inhibit cell proliferation, migration and invasion of RCC cell lines. While a very recent report from Jin et al. (Jin et al., 2017) found a upregulation of miR-30b in RCC tissues and cells. The reason for this difference might due to that the control cell lines in this report is 293 T which is transfected with E1A, however, further investigations are required to figure out the exactly causes.

EMT is a critical driver of cancer metastasis, characterized by the loss of epithelial marker E-cadherin and stimulation of the mesenchymal markers vimentin and N-cadherin, thus inducing migratory and invasive behavior (Pavelic et al., 2011; Satelli et al., 2015). The results of the present study indicated that overexpression of miR-30b-5p induced epithelial marker expression and suppressed the expression of mesenchymal markers. These findings indicated that miR-30b-5p regulated the EMT in RCC cells. The gain in miR-30b-5p also contributed to the inhibition of migration and invasion in the cancer cells by blocking EMT. Therefore, we conclude that miR-30b-5p would be a novel tumor suppressor in RCC due to its negative effect on cell growth and metastasis.

During the process of tumor development, miRNAs may act as oncogenes or tumor suppressors, depending on the target genes. With the results of the present study showing a possible role of miR-30b-5p in RCC pathogenesis as well as knowing that miRNAs could regulate the expression of target genes by interacting with the 3'UTR of their target genes' mRNAs (Chou et al., 2013), we continued investigations to understand the mechanism of miR-30b-5p in tumor pathogenesis. Three bioinformatic algorithms (TargetScan, PicTar, and miRanda) were selected to help find the target gene. Our findings reported here that G-protein subunit α-13 (GNA13) was chosen as the candidate target gene. GNA13 belongs to the G12 subfamily of Guanine nucleotide binding proteins (G proteins) and has been implicated as an oncogenic protein in several human cancers. GNA13 are highly expressed in most aggressive cancer cells (Kelly et al., 2006a; Kelly et al., 2006b). A previous study showed that GNA13 has a critical role of in lysophosphaticid acid (LPA)-stimulated invasive migration of pancreatic cancer cells (Gardner et al., 2013). GNA13 overexpression also drives an aggressive phenotype in human small cell lung cancer and prostate cancer cells and enhances mouse xenograft tumor growth in vivo (Rasheed et al., 2013a; Grzelinski et al., 2010). Another recent study reported that GNA13 is an important mediator of the EMT during colorectal cancer metastasis (Zhang et al., 2014). A recent study also demonstrated that GNA13 has an important role in promoting proliferation and tumorigenicity of gastric cancer (Zhang et al., 2015). It is reported that GNA13 is targeted by multiple miRNAs such as miR-31, miR-182 and miR-200a in cancer (Rasheed et al., 2015; Rasheed et al., 2013b). In the present study, GNA13 was predicted as the target of miR-30b-5p, and overexpression of miR-30b-5p reduced GNA13 mRNA and protein levels in RCC cells. In addition, GNA13 is most highly expressed in RCC cells, and that knockdown of GNA13 suppressed proliferation and metastasis of RCC. Furthermore, re-expression of GNA13 rescued the suppressive effect of miR-30b-5p on the proliferation and metastasis of RCC cells. These results demonstrated that GNA13 plays an important role in the miR-30b-5p-mediated cell proliferation and metastasis inhibition in RCC cells. Collectively, these data suggested that GNA13 functions as a target of miR-30b-5p, which is responsible for miR-30b-5p-mediated regulation of the cell growth and metastasis of RCC.

5. Conclusions

In summary, we investigated the function of miR-30b-5p in RCC growth and metastasis. The results demonstrated that miR-30b-5p was downregulated in RCC and has the ability to inhibit RCC cell proliferation, metastasis and EMT by negatively regulating GNA13. Therefore, miR-30b-5p can function as a tumor suppressor in RCC. These findings support the potential development of miR-30b-5p based prognostic and
therapeutic approaches and improve understanding of the molecular mechanisms underlying RCC development.

Disclosure statement

The authors declare that they have no competing interests.

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