MicroRNA-152 inhibits tumor cell growth by directly targeting RTKN in hepatocellular carcinoma

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Received June 12, 2016; Accepted October 31, 2016

DOI: 10.3892/or.2016.5290

Abstract. Hepatocellular carcinoma (HCC) is the most common form of adult liver cancer and accounts for approximately 90% of all cases of primary liver cancer annually. Rhotekin (RTKN), which functions as a cancer promoter, can be frequently detected in many human cancers, including gastric cancer, colorectal carcinoma and bladder carcinoma. The aim of this study was to investigate the role of RTKN in HCC. Using HCC cells and tissues from patients with liver cancer, we demonstrated that RTKN was significantly increased in HCC. To examine the effect of RTKN on HCC, RTKN overexpressed or silenced HepG2 and Hep3B cells were constructed. Cell proliferation and apoptosis were measured by RT-PCR and flow cytometry. The results showed that RTKN can function as an oncogene and promote the proliferation, while inhibiting apoptosis, of HepG2 and Hep3B cells. Furthermore, we identified that RTKN is a direct gene target of miR-152. miR-152 can reverse the growth promoting effect of RTKN on HCC cells through G2/M phase arrest and nuclear factor-κB (NF-κB) signal inhibition. In conclusion, our research identified that microRNA-152 can inhibit tumor cell growth by targeting RTKN in HCC.

Introduction

Liver cancer is the third leading cause of cancer deaths throughout the world, immediately following lung and colon cancers (1). Hepatocellular carcinoma (HCC) is the most common form of adult liver cancer, accounting for approximately 90% of all cases of primary liver cancer annually (2-4). Treatment of HCC remains a great challenge for clinical medicine worldwide. In the current era of rapid development of anticancer research, it is particularly important to elucidate the tumor biology of HCC, which may provide hope for the development of effective systemic therapy for this disease (5). To this end, the functional mechanisms of many oncogenes or tumor suppressors that may contribute to the development of HCC need to be determined.

Rhotekin (RTKN) is a Rho effector molecule identified in 1996 by yeast two-hybrid screening. RTKN was initially isolated as a scaffold protein with high affinity to Rho-GTP resulting in Rho activation (6,7). According to literature, RTKN is predominantly expressed in human spinal cord and kidney, with a lesser degree of expression in human brain, thyroid, tongue, trachea, liver, stomach and prostate (8). In recent years, upregulated RTKN has been frequently detected in human cancers such as gastric cancer (9), colorectal carcinoma (10) and bladder carcinoma (11), where it functions as a cancer promoter. However, the expression and roles of RTKN in HCC are still unknown and need to be investigated.

MicroRNAs (miRNAs) are short, non-coding RNA molecules that regulate the expression of genes by targeting the 3'-untranslated region (3'UTR) of mRNAs and promoting RNA degradation or interfering with translation (12-14). In recent years, miRNAs have been reported to be involved in multiple biological processes, including proliferation, apoptosis, metastasis and differentiation (14,15). Accumulated studies have demonstrated that aberrant miRNA expression is ubiquitous in the development and progression of multiple human cancers, including gastric carcinoma (16), kidney cancer (17), bladder cancer (18) and HCC (19,20).

In addition, a myriad of studies have suggested that upregulated miRNA commonly act as oncogenic miRNA, while downregulated miRNA generally act as tumor suppressor in cancers (21). Profiling of miRNA expression in human cancers has highlighted microRNA-152 (miR-152) downregulation as a common event in malignancies (22-24) and miR-152 commonly functions as a tumor suppressor by targeting diverse molecules in different cancer cells, such as TGF-α in prostate cancer cells (25), Krüppel-like factor 4 in glioblastoma stem cells (26) and colony stimulating factor-1 in ovarian cancer cell (27). Not surprisingly, miR-152 was also found to be downregulated in human HCC tissues (28) and functioned as a tumor suppressor in HCC cells (29). However, whether RTKN is a target molecule of miR-152 in HCC remains unclear.
In the present study, we detected the expression of RTKN in human liver cancer tissues and hepatocarcinoma cell lines and discovered that RTKN was upregulated in liver cancer samples. Furthermore, we demonstrated that enhanced RTKN could promote HepG2 and Hep3B cell proliferation and inhibit their apoptosis. In vitro, we further identified that RTKN is a direct target of miR-152 in HCC cells, and confirmed that overexpression of miR-152 can reduce RTKN level in HCC cells. Lastly, miR-152 reversed the growth promoting effect of RTKN on HepG2 and Hep3B cells, which is associated with the G2/M phase arrest and NF-kB signal induction induced by miR-152. Hence, we conclude that miRNA-152 can inhibit tumor cell proliferation, but promotes apoptosis by targeting RTKN in HCC.

Materials and methods

Tissue samples. Tumorous liver tissues and corresponding adjacent non-tumor liver tissues were obtained from 20 patients who underwent curative surgery for HCC at the Fourth Military Medical University Affiliated Hospital Tangdu Hospital (Xi'an, China). All subjects were reviewed by a pathologist and histologically diagnosed with HCC. Informed consent was acquired from each recruited patient, none of whom had received chemotherapy or biotherapy treatment before recruitment to this study. The study protocol was approved by the clinical research ethics committee of Tangdu Hospital.

Cell culture. The human HCC cell lines HepG2 (ATCC® HB-8065™), Hep3B (ATCC HB-8064™), SNU-182 (ATCC CRL-2235™) and SNU-449 (ATCC CRL-2234™) were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Human normal liver cell line HL-7702 was purchased from the Chinese Academy of Science Type Culture Collection, Shanghai, China). All cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin (Sigma) and 100 µg/ml streptomycin (Sigma) under a humidified atmosphere of 5% CO₂ at 37°C.

Quantitative reverse transcription polymerase chain reaction. Total RNA was extracted from liver cancer samples (tissues or cells) using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). After quantitation, 4 µg of total RNA was reverse-transcribed by a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) as per the manufacture's instruction. The RT products were used as templates for amplification using the SYBR Green PCR amplification reagent (Qiagen). An Applied Biosystems Prism 7500 Fast Sequence Detection System (Applied Biosystems) was applied to perform the real-time PCR reactions. PCR parameters were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 20 sec. Primers were synthesized by Shanghai Sangon Biological Engineering and Technology Service (China) and shown in Table I. Reactions for each sample were performed in triplicate and the relative levels of target genes were calculated using the 2^{-ΔΔCt} method and normalized to β-actin.

Construction of RTKN overexpressing vector. For RTKN overexpression, the pRK5-Myc-RTKN vector was constructed as previously described (30). Briefly, the full-length cDNA of RTKN was sub-cloned with pRRL3-Myc vector and inserted into a pRKL5-Myc vector. These vectors were transfected into HepG2 or Hep3B cells (96-well plates, 2x10⁴ per well) using Lipofectamine™ 2000 (Invitrogen). Cells transfected with empty pRKL5-Myc vector were used as controls. After transfection for 48 h, RTKN mRNA levels were measured by qRT-PCR. Cell proliferation was detected at 24, 48, 72 and 96 h post-transfection and cell apoptosis was measured at 72 h post-transfection.

RNA interference. For RTKN silencing, a total of 0.2 nmol RTKN siRNA (Cat. no. 143138; Thermo Fisher Scientific, Inc., Waltham, MA, USA) or Silencer® Negative Control no. 1 siRNA (cat. no. AM4611; Thermo Fisher Scientific) was diluted in 2 ml RPMI-1640 medium containing 20 µl lipo-fectamine and incubated at room temperature for 30 min. The above mixtures (100 µl per well) were then added to HepG2 or Hep3B cells in 96-well plates (2x10⁴ cells per well). Finally, cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C. After transfection for 48 h, RTKN mRNA levels were measured by qRT-PCR. Cell proliferation was detected at 24, 48, 72 and 96 h post-transfection and cell apoptosis was measured at 72 h post-transfection.

Vector constructs and luciferase reporter assay. To construct the pGL3-con-RTKN-3'UTR-WT or mutant (Mut) plasmid, the 3'UTR sequence of RTKN predicted to interact with miR-152 or a mutated sequence within the predicted target site, was amplified by PCR and cloned into the XbaI/FseI site of the pGL3-control vector (Promega, Madison, WI, USA). For the luciferase activity analysis, cells were co-transfected with pGL3-con-RTKN-3'UTR-WT or -Mut, a control renilla luciferase pRL-TK vector (Promega), and miR-152 mimic or miR-NC using DharmFECT Duo transfection reagent (Thermo Fisher Scientific). The luciferase activity was detected 48 h later using the Dual-Glo Luciferase assay system (Promega). Data were normalized to the renilla luminescence from the same vector, and deemed as 1 in miR-NC groups.

Co-transfection of miR-152 mimic and pRK5-Myc-RTKN vector. For miR-152 overexpression, miR-152 mimic (cat. no. 4464066) and its negative control (miR-NC; cat. no. 4464058) were purchased from Thermo Fisher Scientific (San Jose, CA, USA). In order to confirm whether miR-152 possesses an inhibitory effect on RTKN expression and function in hepatocarcinoma cells, miR-152 mimic or miR-NC, and pRK5-Myc-RTKN vector were co-transfected into cultured HepG2 and Hep3B cells (96-well plates, 2x10⁴ per well) using Lipofectamine 2000 (Sigma). After transfection for 48 h, RTKN level were detected by RT-PCR and western blotting. Cell proliferation was detected at 24, 48, 72 and 96 h post-transfection and cell apoptosis was measured at 72 h post-transfection.

Western blotting. Cells were first lysed with RIPA buffer. For each sample, protein concentration was measured using the BCA assay (Sigma). Proteins (40 µg) were separated by SDS-PAGE and then transferred onto a PVDF membrane (Sigma). Skim milk powder (0.5%) was used to block the membranes. Target proteins on the membranes were incubated with the appropriate
primary antibodies overnight at 4°C. After washing thrice, the membranes were sequentially incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. The reactive bands were detected by enhanced chemiluminescence (Thermo-Pierce, Rockford, IL, USA) as per the manufacturer’s protocol. The expression of each protein relative to β-actin was analyzed. Antibodies (Abcam, Cambridge, MA, USA) and the dilutions used in this study were as follows: polyclonal rabbit anti-human RTKN antibody (dilution: 1:3000; cat. no. ab154954), polyclonal rabbit anti-human β-actin antibody (dilution: 1:2000; cat. no. ab8227), monoclonal rabbit anti-human cIAP2 antibody (dilution: 1:1500; cat. no. ab32059), monoclonal rabbit anti-human Bcl-xL antibody (dilution: 1:2000; cat. no. ab32370) and HRP-goat anti-rabbit IgG second antibody (dilution: 1:4000; cat. no. ab6721).

Cell proliferation assays. To determine the effects of RTKN on HCC cell proliferation, RTKN was overexpressed or silenced in HepG2 or Hep3B cells using the cell transfection methods described above. Briefly, cells (2x10^4 per well) were seeded in 96-well plates. After transfection, proliferation was examined in the surviving fractions at 24, 48, 72 and 96 h using MTT assay (31). The absorbance was detected at 570 nm wavelength using a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). Each data point was obtained from three independent assays.

Apoptosis assay. After transfection for 72 h, cell apoptosis was analyzed by flow cytometry. Annexin V-FITC Apoptosis Detection Kits (Sigma) were used to stain the cells. Cells were washed twice with Dulbecco's phosphate buffered saline and resuspended in 1X binding buffer at a final concentration of 10^6 cells per ml. Then, 500 µl cell suspension, 5 µl Annexin V-FITC conjugate and 10 µl propidium iodide solution were added into a test tube sequentially. The tubes were incubated at room temperature for 10 min and protected from light. Cells were subsequently analyzed using a FACS analyzer (BD Biosciences, San Jose, CA, USA).

Cell cycle analysis. HepG2 and Hep3B cells were seeded in 10-cm dishes. After synchronizing in serum-free medium for 48 h, cells were harvested and fixed in ice-cold 70% ethanol at 4°C overnight. After washing, cells were treated with propidium iodide (50 mg/ml) and RNase A (100 mg/ml) for 30 min in the dark. Cells were then subjected to flow cytometric analysis to determine the percentage of cells in corresponding phases of the cell cycle (subG1, G0/G1, S, and G2/M). Flow cytometry was performed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with a 488-nm argon laser.

Statistical analysis. Unless otherwise indicated, all experiments were performed in triplicate in three independent experiments. Data are expressed as mean ± standard deviation (SD). Differences between pairs of groups were analyzed by Student's t-test. The values P<0.05 or P<0.01 indicate a statistically significant difference. All the statistical analyses were performed by the SPSS 16.0 software (SPSS; Chicago, IL, USA).

Results

RTKN is upregulated in HCC. To examine the expression of RTKN in liver cancer, HCC tissues and cells were prepared as described above. From the results of 20 pairs of tissue samples,
we found that relative RTKN mRNA levels in cancer tissues were significantly higher than that in normal tissues (Fig. 1A). To further confirm that this change is consistent and common in HCC cell lines, the mRNA level of RTKN in a panel of four HCC cell lines and a normal liver cell line was measured by RT-PCR. As shown in Fig. 1B, RTKN mRNA levels were markedly upregulated in HCC cells (HepG2, Hep3B, SNU-182 and SNU-449) as compared to normal liver cells (HL-7702). These results reveal that the level of RTKN mRNA was universally upregulated in HCC, hinting that increased RTKN mRNA level might contribute to the growth of HCC.

**RTKN accelerates HCC cell growth.** In order to investigate the role of RTKN in HCC cell proliferation and apoptosis, RTKN was overexpressed or repressed in HepG2 and Hep3B cells by transfection with the pRK5-Myc-RTKN/empty vector or RTKN-siRNA/negative control siRNA, respectively. Transfection efficiency was verified by RT-PCR (Fig. 2A). Data from the MTT assay showed that overexpression of RTKN enhanced cell proliferation, while downregulation of RTKN delayed cell proliferation in comparison with the untreated group (Fig. 2B and C). Flow cytometry analysis indicated that RTKN overexpression inhibited cell apoptosis (Fig. 2D and E) and downregulated RTKN accelerated apoptosis (Fig. 2D and F). These results suggest that RTKN functions as an important molecule in regulating the growth of HCC cells.

**RTKN is a direct target gene of miR-152.** Previous studies have demonstrated that miR-152 is downregulated in human HCC tissues (28) and that miR-152 functions as a tumor suppressor in HCC cells (29). MicroRNAs commonly exert their functions by regulating the expression of specific target genes. Through on-line prediction (http://www.microrna.org), RTKN is one of the putative targets of miR-152 with a mirSVR score = -0.1249 < -0.1 (Fig. 3A). To confirm whether or not RTKN can be regulated by miR-152 in HCC cells, luciferase reporter assays were performed in HepG2 and Hep3B cells. The wild-type and mutant 3’UTR of RTKN were constructed and cloned downstream of the luciferase reporter gene. The results demonstrated that miR-152 decreased the luciferase intensity of the wild-type 3’UTR of RTKN by 57.24% in HepG2 cells (Fig. 3B) and 54.94% in Hep3B cells (Fig. 3C) when compared with the miR-NC transfected cells. There was no change in luciferase activity in the mutant groups (Fig. 3B and C). These results indicate that RTKN is a direct target of miR-152. Furthermore, real-time PCR and western blots were used to examine the expression of RTKN in HepG2 or Hep3B cells. As predicted, the mRNA...
and protein level of RTKN in miR-152 mimic and pRK5-Myc-RTKN co-transfected cells was significantly lower than that in pRK5-Myc-RTKN transfected cells (Fig. 3D and E). Together these results indicate that RTKN is a potential target of miR-152 in HCC cells.

miR-152 reverses the growth promoting effect of RTKN on HCC cell. To continue to explore the effect of miR-152 on RTKN functions in HCC cells, we further examined the influences of miR-152 on proliferation and apoptosis of RTKN-overexpressing HepG2 or Hep3B cells. Data shown in Fig. 4A and B suggest that overexpressed miR-152 significantly inhibited the proliferation promoting effect of RTKN on HepG2 and Hep3B cells. Flow cytometry analysis also shows that miR-152 can markedly increase the apoptosis rate of RTKN-overexpressing HepG2 or Hep3B cells (Fig. 4C-E). These results indicate that miR-152 can reverse the growth promoting effect of RTKN on HCC cell by decreasing RTKN expression.

G2/M phase arrest contributes to the inhibition effect of miR-152 on RTKN. Given that miR-152 reverses the growth promoting effect of RTKN on HCC cell, we next tried to determine whether RTKN or miR-152 could influence the cell cycle progression of HCC cell. The results, shown in Fig. 5, reveal that the number of cells in the G2/M phase was markedly increased in miR-152 mimic and pRK5-Myc-RTKN co-transfected cells compared with that in untreated or RTKN-overexpressing cells. This result indicates that miR-152 reverses the growth promoting effect of RTKN on HCC by repressing the cell cycle progression at the G2/M transition in HepG2 and Hep3B cells. The miR-152 induced G2/M phase arrest contributes to the effect of miR-152 on RTKN-overexpressing HepG2 or Hep3B cells.

RTKN-induced NF-κB activation is inhibited by miR-152. It is well demonstrated that NF-κB activation promotes cell survival by switching on the transcription of a series of antiapoptotic genes (32). A previous study reported that RTKN-mediated NF-κB activation confers resistance to apoptosis in human gastric cancer cells (9). In order to further confirm whether miR-152 can regulate the downstream NF-κB signaling of RTKN in HCC, the expression of NF-κB antiapoptotic genes (cIAP-2, Bcl-xl) in HepG2 or Hep3B cells was analyzed by qRT-PCR and western blotting. As shown in Fig. 6A and C, the mRNA of cIAP-2 and Bcl-xl were significantly upregulated in RTKN-overexpressed cells, which was not observed in miR-152 mimic and pRK5-Myc-RTKN co-transfected cells. The same results were observed at the protein level by western blots as shown in Fig. 6B and D. This result suggests that RTKN-mediated NF-κB activation
Figure 4. miR-152 reverses the growth promoting effect of RTKN on HCC. Three groups of HepG2 or Hep3B cells were chosen for analysis of proliferation and apoptosis. Data were analyzed by Student's t-test and expressed as mean ± SD. (A) Proliferation rates of HepG2 cells were detected by MTT assay. *P<0.05 or #P<0.01 vs. untreated and pRK5-Myc-RTKN transfected group; (B) Proliferation rates of Hep3B cells were detected by MTT assay; (C) Cell apoptosis (HepG2 or Hep3B) was analyzed by Annexin V-fluorescein isothiocyanate and propidium iodide (PI) staining. (D) Quantification of relative apoptotic HepG2 cells. (E) Quantification of relative apoptotic Hep3B cells. *P<0.05 or #P<0.01 vs. untreated group.

Figure 5. G2/M phase arrest contributes to the inhibition effect of miR-152. Cells were divided into three groups: Untreated group, pRK5-Myc-RTKN transfected group, pRK5-Myc-RTKN and miR-152 co-transfected group. (A) hepG2 and hep3b cells were stained with propidium iodide and analyzed by flow cytometry. Data were obtained by quantifying the results of flow cytometry, and shown as the cell cycle distribution percentages. A, sub-G1; B, G0/G1; C, S; D: G2/M. (B) Cell cycle distribution of HepG2 cells. (C) Cell cycle distribution of Hep3B cells. *P<0.05 vs. untreated or pRK5-Myc-RTKN-transfected group in corresponding cell cycle phases.
can also be inhibited by miR-152, which can explain the reverse effect of miR-152 on exogenous RTKN-induced HCC cell growth.

Discussion

In this study, we first discovered that RTKN is commonly upregulated in liver cancer tissues and cells, which is consistent with RTKN expression in other cancers, such as gastric cancer (9), colorectal carcinoma (10), and bladder carcinoma (11). To investigate the significance of increased RTKN in liver cancer, RTKN-overexpressing or -silenced HepG2 or Hep3B cells were constructed. Through the MTT assay and flow cytometry analysis, RTKN was found to possess proliferation promoting and apoptosis inhibiting effects on HCC cells, which revealed the carcinogenesis of RTKN in liver cancer. This discovery is in accordance with the role of RTKN in other cancers. For instance, RTKN was found to promote cell proliferation and metastasis in colon cancer (33). Hence, we infer that RTKN can be a potential target molecule for the treatment of liver cancers.

According to previous studies, decreased miR-152 has been commonly found in human cancers (22-24), including liver cancer (28). These studies demonstrated that miR-152 generally functions as a tumor suppressor by targeting diverse molecules in different cancer cells. Through on-line prediction, we discovered that RTKN may be a target gene of miR-152. To examine whether or not miR-152 can regulate RTKN expression in liver cancer, the luciferase reporter assay was performed in HCC cells. Indeed, RTKN is a direct target of miR-152. As expected, miR-152 can decrease RTKN expression in pRK5-Myc-RTKN transfected HCC cells. In human cancers, besides TGF-α, Krüppel-like factor 4 and colony stimulating factor-1, here we identify, for the first time, RTKN as a novel target gene of miR-152.

We next found that miR-152 can reverse the growth promoting effect of RTKN on HCC cells, and that miR-152 can induce cell cycle G2/M phase arrest in pRK5-Myc-RTKN transfected HepG2 or Hep3B cells. Since NF-κB activation promotes cell survival by switching on the transcription of a series of antiapoptotic genes (32) and RTKN-mediated NF-κB activation confers resistance to apoptosis in human gastric cancer cell (9), we suspect that miR-152-induced cell G2/M arrest is associated with the NF-κB signaling pathway. Through the detection of NF-κB antiapoptotic genes/proteins (cIAP-2 and BCl-xL) in HepG2 and Hep3B cells we determined that the expression of cIAP-2 and BCl-xL was markedly increased in RTKN-overexpressing cells, which could be reversed after treating with the miR-152 mimic. Combined with the direct regulation effect of miR-152 on RTKN, we concluded that miR-152 reverses the growth promoting effect of RTKN on HCC cells by inhibiting RTKN-mediated NF-κB activation, which is reflected by miR-152-induced G2/M phase cell cycle arrest.

The Rho proteins are members of the Ras superfamily (34). The diverse functions of Rho are mediated through interacting with its effector proteins. RTKN is a Rho effector protein (8) which can link the Rho signal to NF-κB activation, further leading to increased cell survival by transactivating antiapoptotic genes downstream of NF-κB (8). In this study, we discovered that miR-152 can inhibit the Rho/RTKN/NF-κB signal axis then reverse the growth promoting effect of RTKN
References


