RESEARCH ARTICLE

Effects of miR-152 on Cell Growth Inhibition, Motility Suppression and Apoptosis Induction in Hepatocellular Carcinoma Cells

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Abstract

Background: miR-152 is involved in the genesis and development of several malignancies. However, its role in HCC has not been fully clarified. The aim of this study was to investigate the clinicopathological significance of miR-152 and its effect on the malignant phenotype of HCC cells. Methods: miR-152 expression was detected using real-time quantitative RT-PCR in 89 pairs of HCC formalin-fixed paraffin-embedded and their adjacent tissues. Functionally, in vitro effects and mechanisms of action of miR-152 on proliferation, viability, caspase activity, apoptosis and motility were explored in HepG2, HepB3 and SNU449 cells, as assessed by spectrophotometry, fluorimetry, fluorescence microscopy, wound-healing and Western blotting, respectively. Results: miR-152 expression in HCC was downregulated remarkably compared to that in adjacent hepatic tissues. miR-152 levels in groups of advanced clinical stage, larger tumor size and positive HBV infection, were significantly lower than in other groups. A miR-152 mimic could suppress cell growth, inhibit cell motility and increase caspase activity and apoptosis in HCC cell lines. Furthermore, Western blotting showed that the miR-152 mimic downregulated Wnt-1, DNMT1, ERK1/2, AKT and TNFRSF6B signaling. Intriguingly, inverse correlation of TNFRSF6B and miR-152 expression was found in HCC and bioinformatics confirmed that TNFRSF6B might be a target of miR-152. Conclusions: Underexpression of miR-152 plays a vital role in hepatocarcinogenesis and lack of miR-152 is related to the progression of HCC through deregulation of cell proliferation, motility and apoptosis. miR-152 may act as a tumor suppressor miRNA by also targeting TNFRSF6B and is therefore a potential candidate biomarker for HCC diagnosis, prognosis and molecular therapy.

Keywords: miR-152 - hepatocellular carcinoma - TNFRSF6B - cell growth, motility - apoptosis

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Introduction

MicroRNAs (miRNAs) are endogenously expressed non-coding RNAs that are 20–24 nucleotides long and have been noted to regulate gene expression, cellular differentiation, development and disease (Papagiannakopoulos et al., 2008). Up to now more than a thousand miRNAs have been described in humans (Tsai et al., 2011; Mollaie et al., 2013). MiRNAs regulate various physiological processes, including the stability or translation efficiency of specific mRNAs. As individual miRNAs are capable of regulating a large number of different mRNAs (encoded by 250–500 target genes), there is a strong likelihood that approximately 20–80% of transcribed human genes are regulated by miRNAs. The efficacy in binding and neutralizing their targets depends on various parameters (e.g. primary sequence of the miRNA and target mRNA, three dimensional structure of the miRNA, co-factors, and so on) (Hunt et al., 2009; Tsai et al., 2011; Sun et al., 2013).

Hepatocellular carcinoma (HCC) is a common malignant neoplasm in Asian countries. The estimated number of new cases of HCC has risen to 564,300 and 548,600 patients with HCC have died, representing 97.2% of persons with this diagnosis (Kudo, 2011; Yamazaki et al., 2011; Zhang et al., 2013). The development and progression of HCC involves a multi-step, long-term process. Many reports have highlighted the investigation of genes and proteins underlying the development and progression of HCC (Chen et al., 2006; Chen et al., 2008a; Chen et al., 2008b; Hu et al., 2010; Karabork et al., 2010; Yang et al., 2010; Zhang et al., 2013), however, their sensitivity and specificity remain limited. Therefore, the identification of new biomarkers is urgently needed in order to understand the events causing hepatocarcinogenesis, also to relate various phenotypes

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in clinical features and prognosis.

Previously, we reported that tumor necrosis factor receptor superfamily 6B (TNFRSF6B, also named as Decoy receptor, DcR3/TR6/M68) is overexpressed in both HCC tissue and serum samples compared to other non-malignant liver diseases. TNFRSF6B plays an important role in cell growth and cellular apoptosis of HCC cells in vitro (Chen et al., 2008b; Chen et al., 2010a; Yang et al., 2010). We had predicted the possible miRNAs, which targets TNFRSF6B using different miRNA target prediction software. miR-152 appeared to be one of the potential miRNAs of TNFRSF6B due to the complementary sequences. miR-152 was found to be over-expressed in neuroblastoma cells than in mature neurons (Liu et al., 2012). Meanwhile, inconsistent underexpression of miR-152 was reported in other cancers (Hiroki et al., 2010; Chen et al., 2010b; Tsuruta et al., 2011; Song et al., 2011; Zhou et al., 2012; Chen et al., 2013d; Kohler et al., 2013; Lichner et al., 2013; Song et al., 2013; Azizi et al., 2014), facilitating its role as a tumor suppressor miRNA in these cancers. To our knowledge, only one study with 20 cases of HCC found that the expression of miR-152 was frequently down-regulated in HBV-related HCC tissues in comparison with adjacent noncancerous hepatic tissues (Huang et al., 2010). However, the contribution of miR-152 in the tumorigenesis and progression of HCC has not been fully clarified. The relationship between miR-152 levels and the clinicopathological characteristics has not been reported either. We also hypothesized that TNFRSF6B could be a target gene of miR-152. Thus, in the current study, we investigated the expression of miRNA-152 in HCC and their paired adjacent noncancerous hepatic tissues in 89 cases of formalin-fixed paraffin-embedded (FFPE) surgically resected samples. We also studied the effect of miR-152 on the malignant genotypes of HCC cells in vitro. In addition, we studied the relationship between miR-152 and TNFRSF6B in HCC.

Materials and Methods

Patients

This retrospective study included 89 cases of HCCs and their corresponding paraneoplastic liver FFPE tissues. The ages of HCC patients ranged from 29 to 82 years old, with an average age of 52 years. Clinicopathological information was obtained from medical records and summarized in Table 1. The corresponding paraneoplastic tissues were taken at least 2 cm from the cancerous node. All cases were initial hepatectomies and randomly chosen from the hepatectomies performed in the First Affiliated Hospital, Guangxi Medical University, P.R. China between March 2010 and December 2011. The study protocol was approved by the local Ethical Committee. Written informed consent to use the samples for research was obtained from the patients and clinicians. All samples were independently reviewed and diagnosed by two pathologists.

Real time quantitative RT-PCR (RT-qPCR)

Clinical FFPE blocks were sectioned at a thickness of 10µm (3 sections for total RNA isolation). The total RNA including miRNA was isolated from HCC cells as reported (Dang et al., 2013; Rong et al., 2013a; Rong et al., 2013b). RNA quality and concentration were detected by Nanodrop 2000 (Wilmington, DE 19810 USA). Previously, we found the combination of RUN6B and RUN48 was the best housekeeping gene for HCC FFPE work by using NormFinder and geNorm software (data not shown). Thus, these two genes were used to normalize miR-152 expression in the current study. The primers for miR-152, RNU6B and RNU48 were included in TaqMan® MicroRNA Assays (4427975-000468, Applied Biosystems, Life Technologies Grand Island, NY 14072 USA). The reverse primers were also used in the reverse transcription step with TaqMan® MicroRNA Reverse Transcription Kit (4366596, Applied Biosystems, Life Technologies Grand Island, NY 14072 USA) in a total volume of 10 µl. Real-time qPCR for miRNA was performed with Applied Biosystems PCR7900. The miR-152 abundance in each sample was normalized to its references. The miR-152 expression in FFPE experiment was calculated with the formula 2-ΔΔCq (Chen et al., 2011; Chen et al., 2012; Dang et al., 2013; Rong et al., 2013a; Rong et al., 2013b).

Immunohistochemistry

The procedure of immunohistochemistry for TNFRSF6B staining was as previously described (Chen et al., 2008b; Chen et al., 2010a; Yang et al., 2010). The positive signal for TNFRSF6B appeared yellow brown in the cytoplasm of the cells using 3,3′-diaminobenzidine. One or two of the most representative sections from each case were selected and stained with a rabbit polyclonal antibody against TNFRSF6B (TNFRSF6B-H-130- sc-25464, 1:300 dilution) from Santa Cruz Biotechnology, Inc., Heidelberg, Germany, which is raised against amino acids 171–300 of TNFRSF6B of human origin. One hundred cells from five representative areas from each case were counted. According to immunodetection of stain intensity and number of positive cells, staining results were evaluated by two pathologists, who discussed each case until they reached a consensus. Staining intensity reached the standard of the relative staining intensity of most cells. The degree of staining was subdivided as follows: the staining intensity could range from 0 to 3 (0 = no staining; 1 = yellow or light brown, weak staining; 2 = brown, strong staining; and 3 = dark brown, intense staining), and the positive cells in the liver tissues observed ranged from 0 to 3 in percentage (0, no staining; 1, <30%, often focal or fine granular; 2, 30%–70%, linear or cluster; and 3 >70%, diffuse). Samples were scored by their summation: 0–1 (-); 2–3 (+); 4 (++); 5–6 (+++). Any staining score ≥2 (+) was considered as positive.

Inhibition and re-expression of miR-152 in HCC cells

The human HCC-derived cell lines HepG2 (HB-8065), HepB3 (HB-8064) and SNU449 (CRL-2234) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). They were cultured as previously described (Chen et al., 2010b; Dang et al., 2013; Rong et al., 2013a). All in vitro experiments were
performed in triplicate. HCC cells were planted in 24-well plates (2.5 × 10^4 cells per well) or 96-well plates (2.5 × 10^3 cells per well) and incubated at 37°C for 24 h. The cells were transfected with miR-152 inhibitor, miRNA inhibitor negative control, miR-152 mimic and miRNA mimic negative control, respectively (Ambion, Life Technologies Grand Island, NY 14072 USA) at a final concentration of 200 nmol/L for 96 h using a transfection kit combiMAGnetofection (OZ BIOSCIENCES, Marseille cedex 9 France) in accordance with manufacturer’s procedure.

**Cell biological function detections**

To study the effects of miR-152 inhibitor and miR-152 mimic, cell proliferation, cell viability, apoptosis and nuclear morphology, cell motility and caspase-3/7 activity were performed as described previously (Chen et al., 2010a; Chen et al., 2011; Chen et al., 2012; Chen et al., 2013a; Chen et al., 2013b; Chen et al., 2013c; Dang et al., 2013; Rong et al., 2013). Western blot was performed as described previously (Chen et al., 2010a; Chen et al., 2011; Chen et al., 2012; Chen et al., 2013a; Chen et al., 2013b; Chen et al., 2013c; Dang et al., 2013; Rong et al., 2013). The following primary antibodies were used: Wnt-1 (clone 20C3.1, Merck Millipore), DNMT1 (D59A4, Cell Signaling Technology), phospho-AKT/PKB (pS473, Invitrogen), total-AKT (9272, Cell Signaling Technology), phospho-ERK1/2 (pTpY185/187, Invitrogen), total-ERK1/2 (9102, Cell Signaling Technology), TNFRSF6B (Cell Signaling Technology) and β-actin (Sigma-Aldrich N.V.).
Statistical analysis

SPSS19.0 (Munich, Germany) was used for statistical analysis. Results were representative of minimal three independent experiments. Values were presented as the mean ± standard deviation (SD). Student’s paired or unpaired t-test was performed to analyze significance between paired or unpaired groups. One-way Analysis of Variance (ANOVA) test was used to analyze significance between different groups. The Least Significant Difference (LSD) method of multiple comparisons between two groups was applied when the probability for ANOVA was statistically significant. Correlations were calculated by Spearman’s method. The relationship between miR-152 and recurrence was analyzed by using Kaplan-Meier Survival method. Statistical significance was determined at a \( P<0.05 \) level.

**Results**

**miR-152 expression in HCC FFPE tissues and its clinicopathological significance**

The relative expression of miR-152 in HCC tissues was remarkably lower than that of their matched adjacent noncancerous hepatic tissues \((P<0.001, \text{Table 1, Figure 1A})\). When studying the relationship between miR-152 expression and other clinicopathological parameters, we found that the expression of miR-152 in the tissues of clinical TNM III and IV stages was significantly reduced compared to that in I and II stages \((P=0.013, \text{Figure 1A})\). miR-152 level was also significantly down-regulated in the groups of bigger tumor size and HBV positive infection than the corresponding groups \((P=0.037, P=0.03, \text{Figure 1A})\). miR-152 had no correlation with other characteristics, such as age, gender, histological differentiation grades, metastasis, cirrhosis, plasma AFP level, portal vein tumor embolus, tumor capsular infiltration or tumor nodes \((\text{Table 1})\). Fifty-five of 89 patients were followed up for 3-68 weeks and time-to-recurrence was collected. Time-to-recurrence for the cases of high miR-152 level (higher than the mean) was 59.29±3.41 weeks, longer than that of the low miR-152 group \((31.51±1.80)\). However, this bore no statistical significance. Thus, there was no relationship between the expression of miR-152 and the time-to-recurrence \((P=0.127, \text{Figure 1B})\).

**Effect of miR-152 on the malignant phenotypes in HCC cells**

Transfection efficiency of miR-152 inhibitor and miR-152 mimic was first confirmed using real time RT-qPCR (data not shown). Western blot also confirmed that miR-152 mimic could downregulate the protein expression of two known targets (Wnt-1 and DNMT1 \((\text{Huang et al., 2010; Huang et al., 2014})\)) of miR-152 in HCC \((\text{data not shown})\), which further supported the success of the transfection system. The effect of miR-152 on cell growth was detected using three independent assays, including MTS tetrazolium assay, fluorimetric resorufin viability assay and Hoechst 33342/propidium iodide (PI) double fluorescent chromatin staining, respectively. MTS tetrazolium assay revealed that cell proliferation increased in HepG2 cells from 72 h post transfection compared to blank and negative controls \((* P<0.05, ** P<0.01, \text{compared to blank and negative controls at the same time point})\).
miR-152 Cell Growth Inhibition, Motility Suppression and Apoptosis Induction in Hepatocellular Carcinoma Cells

To validate whether miR-152 is able to influence apoptosis, the CellTiter-Blue assay was multiplexed with a fluorescent caspase-3/7 assay (data not shown) and Hoechst 33342/PI double fluorescent chromatin staining (data not shown), which largely mirrored the results from MTS assay. The effect of miR-152 on cell growth suppression also showed a dose dependent manner in all three cell lines tested (data not shown).

To investigate the contribution of miR-152 in the regulation of cellular signaling, we examined the signaling of ERK and AKT pathways, as well as the proved targets of miR-152 Wnt-1 and DNMT-1 by using western blot, which are related to cell survival, apoptosis and invasion. These pathways were slightly enhanced with miR-152 inhibitor transfection. However, the phospho-ERK1/2 and phospho-AKT were down-regulated notably by miR-152 mimic 96 h post-transfection (data not shown).

We compared the complementarity of miR-152 level and TNFRSF6B protein expression in HCC

![Figure 3. Time Dependent Effect of miR-152 on Cell Caspase-3/7 Activity in HCC Cell Lines.](image)

HepG2, HepB3 and SNU449 cells (2.5×10⁶ cells per well in 96-well-plate) were cultured for 24 h and then transfected with miR-152 inhibitor, miR-152 mimic and their negative controls (200nM) up to another 96 h. Caspase-3/7 activity was detected using Apo-ONE Homogeneous Caspase-3/7 Assay. * P<0.05, ** P<0.01, compared to blank and negative controls at the same time point.

![Figure 4. miR-152 Mimic Suppressed Cell Growth and Induced Apoptosis with Hoechst 33342/Propidium Iodide (PI) Double Fluorescent Chromatin Staining.](image)

HepG2 cells (2.5×10⁶ cells per well in 96-well-plate) were cultured for 24 h then transfected with miR-152 inhibitor, miR-152 mimic and their negative controls (200nM) up to another 96 h. The effect on apoptosis was assessed and compared to mock and negative controls, ×200.
sequence to the 3’-untranslated region of TNFRSF6B with Targetscan (http://www.targetscan.org/), miRTar (http://mirtar.mbc.nctu.edu.tw/human/) and miRanda (http://www.microrna.org/microrna/home.do), respectively. Partial complementarity between miR-152 and TNFRSF6B was observed by all these miRNA target prediction methods (data not shown). Furthermore, the cases of stronger TNFRSF6B expression showed lower miR-152 level (P<0.001). A negative relationship was observed between the miR-152 level and the intensity of TNFRSF6B expression (r=-0.347, P=0.002). The possible relationship was finally verified in vitro. TNFRSF6B protein level was indeed downregulated with the transfection of miR-152 mimic into HepG2 cells for 96 h.

**Discussion**

Aberrant expression of miR-152 has been reported in various classes of malignancies. In neuroblastoma cells, miR-152 was found to be over-expressed compared to mature neurons (Liu et al., 2012). However, underexpression of miR-152 was examined in ovarian cancer (Zhou et al., 2012), bladder cancer (Kohler et al., 2013), prostate cancer (Lichner et al., 2013; Song et al., 2013), endometrial cancer (Hiroki et al., 2010; Tsuruta et al., 2011), pancreatic cancer (Aiziti et al., 2014) and gastrointestinal cancers (Chen et al., 2010c; Song et al., 2011). Thus, the polarity of the implication of miR-152 in malignancies differs between an oncogenic versus a suppressive role in various tumor types. To determine whether miR-152 expression was differentially in human primary liver cancer, Huang et al. (2010) assessed miR-152 expression in 20 pairs of human HBV-related HCC tissues and pair-matched normal hepatic tissues by real-time PCR. Among the 20 HBV-related HCC samples analyzed, the miR-152 levels were significantly decreased in 18 HCC samples (90%) in comparison with the adjacent noncancerous hepatic tissues. In the current study, we found the concordant lowered expression of miR-152 in a larger patient size (89 cases) in HCC, which confirmed that miR-152 expression is frequently downregulated in HCC. This, together with the report of Huang, et al (Huang et al., 2010), suggested that miR-152 may have a tumor-suppressive capacity in the hepatocarcinogenesis and progression of HCC.

Next, we investigated the relationship between miR-152 level and clinicopathological parameters in HCC. Huang, et al (Huang et al., 2010), reported that miR-152 is significantly down-regulated in HBx transgenic mice and HCC cell lines expressing HBx. In the current study, we found the concordant relationship between miR-152 and HBV infection. Lower expression was observed in HBV positive group than in the negative one. We also found that the downregulation of miR-152 was related to the clinical stages of HCC. In the advanced stages III and IV, the miR-152 expression was markedly lower than that in the early I and II stages (P<0.05). Moreover, miR-152 expression was reduced in the group with larger tumor size, which is represented for tumor growth and deterioration. Thus, the result in current study reveals a relation between miR-152 and the progression of HCC. It may be valuable to examine miR-152 expression for the clinical prediction of progression and prognosis of HCC.

Since miR-152 expression is closely related to the HCC progression, next we investigated the contribution of miR-152a to cell growth, apoptosis and motility in HCC cells. The miR-152 mimic decelerated the cell growth and motility in all the cell lines tested (HepG2, HepB3 and SNU449). Additionally, miR-152 mimic enhanced the caspase-3/7 activity and induced apoptosis in HCC cell lines. These results were partially in agreement with Huang, et al (Huang et al., 2010), who also found an inhibition of cell migration and invasion, as well as induced cell apoptosis caused by ectopic miR-152 expression. However, they did not find any significant role for miR-152 in cell proliferation. The contradiction may be due to the products from different company (Gene pharma vs combiMAGnetofection), concentration of mimic (50nM vs 200nM) and incubating time (24h vs 96h). Another study validated that miR-152 inhibition promoted, while miR-152 mimics enhanced cell proliferation caused by ectopic miR-152 expression. However, they did not find any significant role for miR-152 in cell proliferation. The contradiction may be due to the products from different company (Gene pharma vs Ambion), transfection reagent (Lipofectamine 2000 vs combiMAGnetofection), concentration of mimic (50nM vs 200nM) and incubating time (24h vs 96h). Another study validated that miR-152 inhibition promoted, while miR-152 mimics enhanced cell proliferation (Huang et al., 2014), in line with the findings of current study. The biological effect of miR-152 could be explained by the downregulation of the pathways as was shown by western blot, especially ERK1/2 and AKT. Together with the findings of previous reports, the results of current study suggest that the enhanced expression of miR-152 by gene transfection could reverse the malignant phenotypes of HCC cells and these data ascertain a tumor-suppressive role and a prospective therapeutic target of miR-152 for HCC patients.

The mechanisms of miR-152 being a tumor suppressor could be related to different target genes. Diverse target genes of miR-152 have been demonstrated in several cancers. miR-152 has been proved to target the DNMT1 in neuroblastoma (Das et al., 2010), endometrial cancer (Tsuruta et al., 2011), ovarian cancer (Xiang et al., 2014),...
malignant cholangiocarcinoma (Braconi et al., 2010) and in HCC (Huang et al., 2010), which was confirmed in the current study. We also proved that Wnt-1 was another target of miR-152 in HCC, which was consistent with Huang et al. (2014). The cholecystokinin B receptor (CCKBR) (Chen et al., 2010c), colony stimulating factor-1 (CSF-1) (Woo et al., 2012) and transforming growth factor-alpha (TGFα) (Zhu et al., 2013) were also found as putative targets of miR-152 in different cancers (Chen et al., 2010c). However, none of these genes were influenced by miR-152 mimic in HepG2 cells in the current study (data not shown). We have demonstrated the oncogenic role of TNFRSF6B in HCC previously (Chen et al., 2008b; Chen et al., 2010a; Yang et al., 2010). As predicted by several in silico methods for target gene prediction, including Targetscan, miRTar and miRanda, TNFRSF6B was identified as one of the high-scoring candidate genes of miR-152 targets. The TNFRSF6B-encoded mRNA contains a 3′-UTR element that is partially complementary to miR-152, indicating that miR-152 would directly target this site. Then we found TNFRSF6B protein expression was inversely correlated with the levels of miR-152 in HCC. More importantly, with in vitro data, we indeed found that TNFRSF6B protein expression was downregulated by miR-152 mimic transfection.

In conclusions, together with previous reports, the current observations support the notion that miRNA-152 is a tumor suppressive miRNA that plays a role in the oncogenesis and progression of human HCC, by targeting Wnt-1, DNMT1 and TNFRSF6B. miR-152 expression in HCC FFPE samples could be a prognostic biomarker for HCC. Further in vivo test to explore the value of miR-152 as a therapeutic tool on HCC is warranted.

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