Overexpression of microRNA-133b is associated with the increased survival of patients with hepatocellular carcinoma after curative hepatectomy: Involvement of the EGFR/PI3K/Akt/mTOR signaling pathway

XUEDONG WANG1,2, JIANPING ZENG1,2*, LIANG WANG1*, XINJING ZHANG3, ZHIQIAN LIU2, HONGYI ZHANG1,3 and JIAHONG DONG1,2,3

1Department of Hepatobiliary Surgery, Beijing Tsinghua Changgung Hospital, Medical Center, Tsinghua University, Beijing 102218; 2Department of Hepatobiliary Surgery, Chinese General PLA Hospital, Beijing 100853; 3Medical School of Tsinghua University, Beijing 100084, P.R. China

Received June 25, 2016; Accepted August 9, 2016

DOI: 10.3892/or.2017.5699

Abstract. The aim of the present study was carried out to investigate the association of microRNA-133b (miRNA-133b) expression with the prognosis of patients with hepatocellular carcinoma (HCC) after curative hepatectomy. In the present study, the expression of miRNA-133b in HCC tissues was determined to be lower than that noted in the adjacent normal tissues. Overall and disease-free survival of the HCC patients with high miRNA-133b expression was observably extended, compared with the HCC patients with low miRNA-133b expression. Moreover, the overexpression of miRNA-133b inhibited cell proliferation, increased lactate dehydrogenase (LDH) activity, induced apoptosis and promoted caspase-3/8 activities and Bax/Bcl-2 protein expression in HCC cells, whereas the protein expression of epidermal growth factor receptor (EGFR) was significantly decreased. The overexpression of miRNA-133b significantly suppressed PI3K, phosphorylated (p)-Akt and p-mTOR protein expression in HCC cells. GW2974, an EGFR inhibitor, suppressed the protein expression of EGFR, inhibited cell proliferation, increased LDH activity, induced apoptosis and promoted caspase-3/8 activities and Bax/Bcl-2 protein expression, downregulated PI3K, p-Akt and p-mTOR protein expression in the transfected HCC cells overexpressing miRNA-133b. Taken together, our results indicate that the overexpression of miRNA-133b is associated with the increased survival of HCC patients after curative hepatectomy. The effects of miRNA-133b in HCC are mediated through the EGFR/PI3K/Akt/mTOR signaling pathway.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors (1). According to data since 2010, HCC ranks fifth among the most commonly diagnosed malignant tumors in men and eighth in women (1). The number of patients diagnosed with HCC has continued to increase (2). The disease can be radically cured by surgical removal; the first choice for HCC treatment in China and many other countries (3). Through ultrasound screening for patients with liver cirrhosis or hepatitis, numerous patients suitable for hepatectomy have been identified (4). Moreover, with the development of surgical technology and preoperative management, hepatectomy for HCC has become a safe surgery with rare complications (4). Over the past 30 years, the survival rate of HCC patients who have undergone hepatectomy has been markedly improved (5). Nevertheless, HCC mainly follows the occurrence of viral hepatitis and liver cirrhosis in China and Southeast Asia (6). The long-term effects are unsatisfactory. Postoperative occurrence is the primary cause of treatment failure (7).

Neoplasm staging is of great significance (5). It is conducive to the development of treatment strategies and the prognostic assessment of patients (8). However, it provides standardized platforms for the evaluation of new treatment methods and the comparison of curative effects. As for HCC, there are many treatments to this disease, including surgical re-resection, TACE, PEIT, RFA, microwave therapy, radiotherapy, cryotherapy and liver transplant (9). Wherein, surgical re-resection is still the most effective treatment (9). The appropriate treatment should be selected according to the clinicopathological features of the recurrent carcinoma, characteristics of the patients, reserve functions of the liver and the general conditions of the patients (7).
Recurrence and metastasis are important factors influencing the curative effects. Since the time of intrahepatic recurrence affects the survival rate of patients after recurrence, there is an urgent need for indicators able to accurately predict the postoperative prognosis of HCC patients (10). Currently, there are numerous studies concerning the prognostic factors of HCC after surgical resection both at home and abroad (11). These factors include clinicopathological factors of the primary and recurrent tumors, the origin of HCC, time of recurrence, general conditions of the patients and surgical methods (11). However, due to the different liver diseases and non-uniform standard for radical resection, there are differences in the markers influencing the postoperative prognosis of HCC patients (12).

The mature microRNA (miRNA) is a type of non-coding and single-stranded RNA molecule with 19-25 nucleotides (10). The precursor of miRNA is referred to as pri-miRNA. It is double-stranded RNA consisting of 70-100 nucleotides. After cutting and unwinding, it develops into mature miRNA (13). miRNA sequences are highly conserved among different species, which indicates that these molecules play an important role in the development, proliferation, differentiation and apoptosis in organisms (13). However, a number of studies suggest that there are obvious differences in the miRNA profile in different diseases and on gene expression (13,14). This indicates that certain miRNAs can be regarded as biomarkers and used as diagnostic and prognostic indicators of malignant tumors (15).

A large amount of research results have shown that the overexpression or mutation of epidermal growth factor receptor (EGFR) exists in many tumor cells and tumor tissues (16). EGFR refers to the transmembrane receptor with RTK activity. It is coded by c-erbB1 genes. The ligands of EGFR include EGF, TGF, AREG, HB-EGF, EPR and β-cytokine (17). It regulates the biological activity of cells by mediating various signaling pathways (18). The overexpression or mutation of EGFR is often associated with the poor prognosis, rapid metastasis, short-term recurrence and short survival time of epithelium tumors such as breast, gastrointestinal, ovarian and cervical cancer (19).

PI3K is a kinase that is involved in multiple cellular signaling pathways. As regards Akt, it is an important factor in the downstream of PI3K (20). It is able to regulate cell proliferation and prevent cell apoptosis. PI3K/Akt is one of the principal downstream signaling pathways of the ERBB family of receptor tyrosine kinases (21). The cytoplasmic domain of ERBB3 activates PI3K through the phosphorylation of tyrosine. The activation of a catalytic subunit of PI3K leads to the excessive activation of PI3K in a variety of tumor tissues (22).

Akt is a serine/threonine-specific protein kinase. Since it is highly homologous with PKA and PKC, it is also referred to as protein kinase B (PKB) (23). It plays an important role in the regulation of cell growth, proliferation and survival and glucose metabolism. AKT is able to inhibit Smac/DIABLO, activate and upregulate apoptosis-inducing factors and inhibit p53 with Mdm2 (23). In addition, it enables the inhibitory factors of CDK (p21CIP1/WAF1 and p27KIP1) to move out of the cell nucleus and degrade in the cytoplasm through phosphorylation, thereby promoting cell proliferation.

mTOR is an important site in the downstream of the PI3K signaling pathway. It plays a critical role in tumor development, invasion, metastasis and angiogenesis. In 90-100% of HCC cases, the AKT/mTOR signaling pathway is activated, which is significantly correlated with the recurrence of tumors and reduced survival rate of patients (21). In this study, we evaluated the possible associations between microRNA-133b (miRNA-133b) and the prognosis of patients with HCC in light of clinicopathological characteristics after curative hepatectomy, and investigated the effect of miRNA-133b on the EGFR/PI3K/Akt/mTOR signaling pathway.

Materials and methods

Patients and follow-up. We analyzed the prospective collected data of 112 HCC patients after curative hepatectomy between February 2010 and May 2011 at the Chinese General PLA Hospital. The present study was approved by the Institutional Ethics Committee of the Chinese General PLA Hospital. Written informed consent was obtained from all patients. Basic clinical characteristics of all patient with HCC were collected and are documented in Table I. During the first 2 years after surgery, patients were followed-up every 2 months, and from 3 years after surgery, patients were followed-up every 3-4 months.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). The miRNA-133b expression levels in HCC tissues and matched adjacent normal tissues were detected using qRT-PCR. Total RNA was extracted from HCC tissue samples and adjacent normal tissues using TRIzol (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instructions. Specific cDNA was synthesized from total RNA (5-10 ng) using TaqMan MicroRNA assays protocol (Applied Biosystems, Foster City, CA, USA). qRT-PCR (7900HT Fast Real-Time PCR System) was performed using GeneAmp® Fast PCR Master Mix (both from Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA) to analyze the expression level of miRNA-133b. The qPCR conditions were as follows: 95˚C for 5 min; 40 cycles of 94˚C for 30 sec; 60˚C for 45 sec; 72 for 45 sec. The following primers were used: miR-133b forward, 5'-GAACCAAGCCGCCGAGA-3' and reverse, 5'-CGCCCTGTCTGTGCTGGT-3'; RNU6B was used as internal control: U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCAGATTTTCGCTGCA-3'. Relative quantification of miRNA-133b expression was evaluated using the formula, $2^{-\Delta\Delta C_t}$.

Cell culture. Human HCC cell lines HepG2, SMMC7721, Bel7404 and HCCM3 were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (both from Invitrogen; Thermo Fisher Scientific, Inc.) 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37˚C.

miRNA transfection. The HepG2 cells (1x10^5 cells/well) were seeded into 6-well plates and cultured overnight. Then, the cells were transfected with 100 nM of negative control miRNA or the miR-133b mimics (both from Shanghai GenePharma Co., Ltd., Shanghai, China) using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.). At 48 h post-transfection, the
transfected HepG2 cells were prepared for further analysis. EGFR inhibitor (GW2974 2 µM Sigma Chemical Co. St. Louis, MO, USA) was added into cell after transfection at 4 h.

**Cell viability assay.** The transfected HepG2 cells (1x10⁴ cells/well) were seeded into 96-well plates and cultured overnight. Cell viability was cultured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Invitrogen; Thermo Fisher Scientific, Inc.) assay for 4 h in a humidified atmosphere of 5% CO₂ at 37˚C. The medium was replaced, and then dimethyl sulfoxide (DMSO) (150 µl) was added into every well and shaken for 20 min at 37˚C. Absorbance was measured using the EL800 Universal Microplate Reader at 570 nm.

**Measurements of lactate dehydrogenase (LDH) activity.** The transfected HepG2 cells (1x10⁴ cells/well) were seeded into 96-well plates and cultured overnight. Cells were washed with ice-cold phosphate-buffered saline (PBS) and obtained by resuspending the cells in RIPA buffer kit (Beyotime Institute of Biotechnology, Shanghai, China). The protein concentration was determined using the Bradford protein assay kit (Beyotime Institute of Biotechnology). Protein (50 µg) was subsequently separated using 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes (EMD; Millipore, Billerica, MA, USA). The membranes were blocked using 5% skim milk powder and incubated overnight with primary antibodies: anti-Bax, anti-Bcl-2, anti-PI3K, anti-p-Akt, anti-p-mTOR and GAPDH at 4˚C overnight. The membranes were then washed with Tris-buffered saline with Tween-20 (TBST) and incubated with HRP-conjugated goat anti-rabbit immunoglobulin G for 1 h at room temperature. Protein bands were visualized with the Chemi-Lumi One L western blotting substrate.

**Statistical analysis.** All data are presented as the mean ± standard error. The Kaplan-Meier method was used to estimate survival rates, and the log-rank test was used to assess survival differences between groups. Data from each group were statistically analyzed using the Student’s t-test. Differences were considered statistically significant at a p-value of <0.05.

**Results**

**Expression of miRNA-133b in patients with HCC after curative hepatectomy.** We firstly assayed the expression of miRNA-133b in patients with HCC after curative hepatectomy. As shown in Fig. 1, the expression of miRNA-133b in the HCC tissues was effectively lower than that in the adjacent normal tissues. We then assessed that the correlation between miRNA-133b expression and clinicopathological features of HCC and found that the tumor size of the HCC patients with low miRNA-133b expression was larger than that of the HCC.
patients with high miRNA-133b expression (Table II). The Edmondson grade and serum AFP levels of the HCC patients with low miRNA-133b expression were also higher than these parameters in the HCC patients with high miRNA-133b expression (Table II).

Effects of miRNA-133b expression on overall and disease-free survival of HCC patients. We aimed to ascertain the affects of miRNA-133b expression on the overall and disease-free survival of HCC patients. As shown in Fig. 2, the overall and disease-free survival of HCC patients with high miRNA-133b expression was observably extended, compared with the HCC patients with low miRNA-133b expression.

Expression of miRNA-133b in HCC cell lines. We detected the expression of miRNA-133b in HCC cell lines using RT-qPCR. As shown in Fig. 3, the expression of miRNA-133b in the HepG2 cells was lowest among the HCC cell lines (HepG2, SMMC7721, Bel7404 and HCCM3). Thus, we selected the HepG2 cells for use in further experiments.

Overexpression of miRNA-133b inhibits the proliferation of and increases LDH activity in HepG2 cells. We investigated the effects of miRNA-133b on cell proliferation and LDH activity in HepG2 cells. As shown in Fig. 4, the overexpression of miRNA-133b significantly inhibited the proliferation of and increased LDH activity in the HepG2 cells, compared with these parameters in the negative group.

Overexpression of miRNA-133b induces the apoptosis of HepG2 cells. We confirmed the effects of miRNA-133b on the apoptosis of HepG2 cells. As shown in Fig. 5, the overexpression of miRNA-133b significantly induced the apoptosis of the HepG2 cells, compared with negative group.

### Table II. Correlation between microRNA-133b expression and clinicopathological features of HCC.

<table>
<thead>
<tr>
<th>Variables</th>
<th>All patients (n=112)</th>
<th>Low miRNA-133b</th>
<th>High miRNA-133b</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤55</td>
<td>63</td>
<td>35</td>
<td>28</td>
<td>0.892</td>
</tr>
<tr>
<td>&gt;55</td>
<td>49</td>
<td>23</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td>0.933</td>
</tr>
<tr>
<td>Female</td>
<td>58</td>
<td>31</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>54</td>
<td>28</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>≤3.0</td>
<td>49</td>
<td>35</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>&gt;3.0</td>
<td>63</td>
<td>48</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Edmondson grade</td>
<td></td>
<td></td>
<td></td>
<td>0.011</td>
</tr>
<tr>
<td>I</td>
<td>12</td>
<td>7</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>67</td>
<td>48</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>33</td>
<td>25</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Serum AFP levels (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>75.2±16.8</td>
<td>108.2±301.2</td>
<td>53.7±167.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>38.2±4.5</td>
<td>39.9±6.1</td>
<td>37.3±5.9</td>
<td>0.371</td>
</tr>
<tr>
<td>Bilirubin (µmol/l)</td>
<td>19.4±11.2</td>
<td>18.3±10.2</td>
<td>20.6±10.8</td>
<td>0.782</td>
</tr>
</tbody>
</table>

HCC, hepatocellular carcinoma.

Figure 2. Effects of microRNA-133b expression on overall and disease-free survival of hepatocellular carcinoma patients. Effects of microRNA-133b expression on (A) disease-free and (B) overall survival of hepatocellular carcinoma patients.
Overexpression of miRNA-133b promotes caspase-3/-8 activities and increases the Bax/Bcl-2 protein expression ratio in HepG2 cells. To explore the mechanisms underlying the induction of apoptosis in the HepG2 cells by miR-133b, we determined caspase-3/-8 activities and Bax/Bcl-2 protein expression in the HepG2 cells. As shown in Fig. 6A-D, the overexpression of miRNA-133b significantly promoted caspase-3/-8 activities and increased the Bax/Bcl-2 protein expression ratio in the HepG2 cells, compared with the negative group.

Overexpression of miRNA-133b suppresses EGFR protein expression in HepG2 cells. To verify whether EGFR is a direct target of miRNA-133b, we measured EGFR protein expression by western blot analysis. As shown in Fig. 6C and E, the overexpression of miRNA-133b significantly suppressed EGFR protein expression in the HepG2 cells, compared with the negative group.

Overexpression of miRNA-133b suppresses PI3K, p-Akt and p-mTOR protein expression in HepG2 cells. Moreover, we verified that the PI3K/Akt/mTOR signaling pathway is a direct target of miRNA-133b. We assessed PI3K, Akt and mTOR protein expression by western blot analysis. As shown in Fig. 7, the overexpression of miRNA-133b significantly suppressed PI3K, p-Akt and p-mTOR protein expression in the HepG2 cells, compared with the negative group.

EGFR inhibitor enhances the suppressive effects on EGFR protein expression in HepG2 cells induced by the overexpression of miRNA-133b. To further investigate the role of EGFR in the effects of miRNA-133b on the poor of patients with HCC after curative hepatectomy, we downregulated EGFR expression using a EGFR inhibitor (GW2974, 2 µM). As shown in Fig. 8A and B, GW2974 significantly downregulated EGFR expression in the miRNA-133b-overexpressing HepG2 cells when compared with the miRNA-133b overexpression only group.

EGFR inhibitor enhances the inhibitory effects on cell proliferation induced by the overexpression of miRNA-133b and increases LDH activity even further in HepG2 cells. Furthermore, we investigated the effects of the downregulation of EGFR on the effects of miRNA-133b on the poor of
patients with HCC after curative hepatectomy. As shown in Fig. 9, the downregulation of EGFR significantly inhibited cell proliferation and increased LDH activity in the miRNA-133b-overexpressing HepG2 cells compared with the miRNA-133b overexpression only group. **EGFR inhibitor enhances the promoting effects on the apoptosis of HepG2 cells induced by the overexpression of miRNA-133b.** To further confirm whether the downregulation of EGFR mediates the promoting effects of miRNA-133b on the apoptosis of HepG2 cells, the apoptosis rate of HepG2 cells
was measured by flow cytometry. As shown in Fig. 10, the downregulation of EGFR significantly induced the apoptosis of the miRNA-133b-overexpressing HepG2 cells, compared with the miRNA-133b overexpression only group.

**EGFR inhibitor enhances the promoting effects on caspase-3/-8 activities and on the Bax/Bcl-2 protein expression ratio in HepG2 cells induced by the overexpression of miRNA-133b.** In HCC cells, when EGFR expression was downregulated, the effects on the apoptosis of HepG2 cells overexpressing miRNA-133b were assessed. As shown in Figs. 8A and C, and 11, the downregulation of EGFR significantly promoted caspase-3/-8 activities and increased the Bax/Bcl-2 protein expression ratio in the miRNA-133b-overexpression group.

---

**Figure 8.** EGFR inhibitor suppresses EGFR and Bax/Bcl-2 protein expression ratio in HepG2 cells by overexpression of microRNA-133b. GFR inhibitor suppressed EGFR and Bax/Bcl-2 protein expression ratio using (A) western blot and (B and C) statistical analyses in HepG2 cells by overexpression of microRNA-133b; “p<0.01 compared with the negative group; ”p<0.01 compared with the microRNA-133b overexpression only group.

**Figure 9.** EGFR inhibitor inhibits cell proliferation and increases LDH activity in HepG2 cells by overexpression of microRNA-133b. EGFR inhibitor (A) inhibited cell proliferation and (B) increased LDH activity in HepG2 cells by overexpression of microRNA-133b; “p<0.01 compared with the negative group; ”p<0.01 compared with the microRNA-133b overexpression only group.

**Figure 10.** EGFR inhibitor induced the apoptosis of HepG2 cells by overexpression of microRNA-133b; “p<0.01 compared with the negative group; ”p<0.01 compared with the microRNA-133b overexpression only group.
expressing HepG2 cells compared with the miRNA-133b overexpression only group.

**EGFR inhibitor enhances the suppressive effects on PI3K, p-Akt and p-mTOR protein expression in HepG2 cells induced by the overexpression of miRNA-133b.** To examine whether EGFR regulates miRNA-133b in regards to HepG2 cell growth, we suppressed EGFR expression and then analyzed the effects on the PI3K/Akt/mTOR signaling pathway. As shown in Fig. 12, the downregulation of EGFR significantly downregulated the PI3K/Akt/mTOR signaling pathway in miRNA-133b-overexpressing HepG2 cells compared with the miRNA-133b overexpression only group.

**Discussion**

Hepatocellular carcinoma is one of the most common malignant tumors. Its incidence is increasing. Due to the technological development of hepatic surgery and the establishment of new treatments, the disease-free survival of patients has been extended (24). However, the recurrence rate remains high. Various foreign documents report that the recurrence rate of HCC can reach 70-80% within 5 years after partial hepatectomy. According to domestic documents, the recurrence rate can be up to 57-81% within 3 years after hepatectomy. The results of the present study showed that the overexpression of miRNA-133b inhibited cell proliferation, increased LDH activity, induced apoptosis and promoted caspase-3/-8 activities and Bax/Bcl-2 protein expression in HepG2 cells. The activation or mutation of EGFR may cause the cascade of signaling pathways downstream and finally induce uncontrollable proliferation of tumor cells (19). The overexpression of miRNA-133b inhibited cell proliferation, increased LDH activity, induced apoptosis and promoted caspase-3/-8 activities and Bax/Bcl-2 protein expression in HepG2 cells.

The activation or mutation of EGFR may cause the cascade of signaling pathways downstream and finally induce uncontrollable proliferation of tumor cells (19). The overexpression of miRNA-133b inhibited cell proliferation, increased LDH activity, induced apoptosis and promoted caspase-3/-8 activities and Bax/Bcl-2 protein expression in HepG2 cells.

The excessive activation of PI3K plays a critical role in the occurrence and development of HCC (26). There are a number of different mechanisms mediating the upregulation of PI3K expression. LMP1 is able to activate PI3K directly, thereby causing the phosphorylation of Akt (26). With the activation of NF-κB, the TRAF binding domain in LMP1 becomes
the active site (20). The activation of the phosphorylation of Akt can further induce the phosphorylation of downstream molecules (27). In addition, it may make them participate in cellular metabolism, proliferation, survival and growth. Upstream molecules of Akt are blocked by PDK1, which effectively inhibit the growth of tumor cells. Most HCC cases have sustained activation (26). The phosphorylation and overexpression of Akt can be detected in HCC tissues and HCC cell lines.

Activated mTOR can regulate key factors of protein translation such as p70S6K and 4EBP1 (28). The latter relieves the depression of eIF4E and finally induces the translation of a series of proteins that promote cell growth. EIF4E gene amplification and overexpression are closely linked with the clinical progression of HCC (29). EMT is obviously inhibited in HCC with high expression of high mobility group box 1 (hmgb1) predicts poor prognosis for hepatocellular carcinoma after curative hepatectomy. Tumour Biol 34: 271-276, 2013.

In conclusion, our results suggest that the overexpression of miRNA-133b increases the survival of patients with HCC after curative hepatectomy through the EGFR/Pi3K/Akt/mTOR signaling pathway.

Acknowledgements

The present study was supported by the First-Class General Financial Grant from the China Postdoctoral Science Foundation (grant no. 2014M562551).

References


