Upregulation of miR-650 is Correlated With Down-Regulation of ING4 and Progression of Hepatocellular Carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the high occurrence lethal cancers in China [1,2], which accounts for 85–90% of all primary liver cancers and ranks as the fifth most prevalent malignancy worldwide [3]. Relapse and metastasis are frequently observed in the clinic for HCC patients, and the 5-year survival rate remains quite low because of the late diagnosis and lack of effective novel therapeutic tools. Although the intensive studies about HCC has been conducted currently, but the tumorigenesis mechanism of HCC still remains unclear. Therefore, it is crucial to discover the novel molecular targets which may be used in the development of more effective therapy methods or to facilitate early diagnosis for HCC patients.

miRNAs are small noncoding single-stranded RNAs which have been reported frequently in expression regulation of genes by binding to target miRNAs. They have been implicated in cancer development, cell proliferation, apoptosis, and differentiation. Recently, many miRNAs were found to be frequently deregulated or upregulated in HCC, and some specific miRNAs were found to be associated with the clinicopathological features of HCC, such as metastasis, recurrence, and prognosis [4–6]. Moreover, compelling evidence demonstrated that miRNAs played important roles in HCC progression and directly contribute to cell proliferation, avoidance of apoptosis, and metastasis of HCC.

miR-650, a novel miRNA, is reported to be upregulated in gastric cancer. It promotes proliferation of tumor cells, partially through direct binding of its target ING4 [7]. miR-650 level was significantly correlated with acral melanomas [8]. It has also been reported that p16(INK4a) could induce the expression of miR-650 in MCF7 cells and miR-650 could down regulate CDK1 by combining with CDK1-3’UTR [9]. However, related research about miR-650 has not yet been reported in HCC.

In this study, the expression of miRNA-650 has been evaluated in 120 paired HCC tissues and PCL tissues. To further investigate the relationship between miR-650 and clinicopathological factors, a total of 248 HCC specimens were analyzed. Furthermore, we found a negative correlation between miRNA-650 and ING4. It implies that miRNA-650 and its target are essential for HCC progression. It may provide a potential novel diagnostic factor for HCC and provide novel therapeutic opportunities for HCC treatment.

MATERIALS AND METHODS

Tissues

In this study, 120 paired HCC tissues and paracarcinomatous liver (PCL) tissues and the other 128 HCC tissues were obtained from the Second Affiliated Hospitals of Harbin Medical University (Harbin, China), from 2007 to 2009. The normal and tumor states of specimens were confirmed by examination of hematoxylin and eosin-stained histology sections by pathologists. Histological tumor subtypes were assessed with the World Health Organization classification. Histopathological grading of tumors into well, moderate, or poorly differentiated types was performed in a qualitative manner based on conventional pathological criteria (i.e., architectural and

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cytological atypia). Tumors were staged by American Joint Commit-
tee on Cancer Tumor Node Metastasis classification system [10]. Sur-
geous tissues were collected and immediately frozen in liquid ni-
trogen, which were subsequently stored at −80 °C for RT-PCR and
western blotting analysis. All patients provided written, informed
consent with procedures approved by the Human Ethics Review
Board.

Cell Culture and Proliferation Assay
Normal liver cells THLE-2 (CRL-2706, ATCC) were cultured in
medium BEGM and 10% FBS that was added extra 5 ng/ml EGF,
70 ng/ml Phosphoethanolamine at 37 °C in a humidified atmosphere
of 5% CO₂. Cells were transfected with 50 nM miRNA duplex
(RIBOBIO, China), scrambled duplex (negative control, RIBOBIO),
or 200 nM miRNA inhibitor (single antisense sequence of miRNA,
RIBOBIO) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).
THLE-2 cells (5 × 103 per well) were plated in 96-well plates and
cell proliferation was measured using the MTT assay (G3580, Prom-
ega, Madison, WI).

RNA Extraction, Reverse Transcription, Primers, and
Real-Time PCR Reaction
Total RNA was isolated from tissues using the miRNeasy FFPE
Kit (Qiagen, Germantown, MD) and cDNA synthesis was performed
using the First-Strand cDNA Synthesis Kit (Promega) following the
manufacturer’s protocol. The one-step SYBR Green I-based real
time polymerase chain reaction (real-time PCR) was performed to
detect mature miR-650 and ING4 expression levels in 248 samples
(one-step SYBR RT-PCR kit; TaKaRa, Dalian, China). PCR primer
sequences were as follows: for ING4, forward 5'-CTTACAGGG-
GAGGTCC TTTT-3' and reverse 5'-GCCAGAGCCTAGATGACCTG
-3'; for β-actin, forward 5'-TGGCACCACGCAATGAA-3' and re-
verse 5'-CTAAGCTAATGGGCTCTAA-3'; the first-strand primer for
miR-650 used in reverse transcription is 5'-GTCGTATCC-
AGCGTGTTGCTGAGTGGCAATTGCACTGGATACGACG-
GTCGTATCC-3' and reverse 5'-GTCGTATCCAGCGTGTTGCTGAGTGGCAATTGCACTGGATACGACG-
GTCGTATCC-3'. Expression level was calibrated by HCC/PCL. It was shown as Figure 1B that
miR-650 level is significantly high in age ≤60 (P = 0.041) and
poor/moderate differentiation (P = 0.017). Furthermore miR-650
level is higher in stage III–II than stage I, also in lymph node metas-
tasis N2–N1 than N0, lower in tumor size less 30 mm, but no statisti-
cal significance.

The Correlation Analysis Between miR-650 Expression
Level and Clinicopathological Factors in HCC Patients
To further investigate the relationship between miR-650 and
HCC, 248 HCC tissues were analyzed. Expression level of miR-650
was examined by quantitative real-time PCR in these 248 HCC
tissues and using U6 as a native control. Wilcoxon test was used to
analyze the correlation between miR-650 and clinicopathological
factors, such as gender, age, tumor size, stage, lymph node metasta-
sis, and the extent of differentiation.

We found that the expression level of miR-650 significantly corre-
lated with age (stronger in ≤60 years old than >60, P = 0.0019),
tumor stage (greater in II/III than I, P = 0.0069), and differentiation
status (more stronger in poorly differentiated samples than well-
differentiated or moderately differentiated samples, P = 0.0108), as
shown in Table I. However, there is no correlation between miR-650
expression and gender, tumor size, or lymph node metastasis.

miR-650 May Be Involved in HCC Genesis by
Down-Regulating ING4 Expression
To demonstrate the role of miR-650 in HCC, we first performed
RT-PCR for miR-650 and western blot for ING4 in eight HCC
tissues (T) and three normal liver tissues (N) as control. PCR prod-
ucts run on gels and evaluated by gray value with the software Image
J. The cutoff value was set by mean gray value of miR-650, less
than mean was regarded as negative, shown by ‘’−’’ and over mean
was regarded as positive, shown by ‘’+’’. Thus, miR-650 expres-
sion was negative in T3, T5, T6, and T11, while the other samples
were positive. It demonstrated that miR-650 level is weaker in
normal tissues than in tumor tissues, however, ING4 levels were
higher in these same tissues. In tumor tissues, the expression of miR-
650 and ING4 also display a negative correlation type (Fig. 2A).
Furthermore, we compared the miR-650 and ING4 expression in 122 HCC tissues by real-time PCR. The data are consistent with normal distribution but the variance is not consistent, so Spearman test was used to evaluate the correlation. The correlation coefficient is $r = 0.2011$ ($P = 0.0264$) and the scatter diagram is shown as Figure 2C. The statistical analysis data suggested that there is a significant correlation between miR-650 and ING4.

**miR-650 Decrease ING4 Level and Promote Liver Cells Proliferation**

To further study the mechanisms of miR-650 in HCC, we first investigated its inhibition role to ING4 in normal liver cells THLE-2, then the correlation between miR-650 and cell proliferation was evaluated by MTT assay. Results suggested that overexpressed miR-650 significantly inhibit the expression of ING4 (Fig. 3A,B) and significantly stimulated cell viability at 72, 96, 120, and 144 hr (Fig. 3C).

**DISCUSSION**

HCC is a fatal disease that carries a poor 5-year prognosis. Current findings demonstrate that many miRNAs are differentially expressed in HCC, and have essential roles in liver cancer progression by directly targeting a large number of critical genes in HCC cells [11]. For example, miR-122 accounts for 70% of the total liver
TABLE I. Correlation between miR-650 and clinicopathological factors of HCC

<table>
<thead>
<tr>
<th>Factors</th>
<th>Number</th>
<th>Expression of miR-650 (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>144</td>
<td>2.53 ± 0.48</td>
</tr>
<tr>
<td>Female</td>
<td>104</td>
<td>2.34 ± 0.47</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>79</td>
<td>3.36 ± 0.84</td>
</tr>
<tr>
<td>&gt;60</td>
<td>169</td>
<td>2.20 ± 0.36</td>
</tr>
<tr>
<td>Tumor size (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤30</td>
<td>115</td>
<td>2.36 ± 0.41</td>
</tr>
<tr>
<td>&gt;30</td>
<td>133</td>
<td>2.52 ± 0.53</td>
</tr>
<tr>
<td>Stage</td>
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<td></td>
</tr>
<tr>
<td>I</td>
<td>86</td>
<td>0.95 ± 0.23</td>
</tr>
<tr>
<td>II</td>
<td>114</td>
<td>2.40 ± 0.49</td>
</tr>
<tr>
<td>III</td>
<td>48</td>
<td>2.80 ± 0.55</td>
</tr>
<tr>
<td>Node</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0/N1</td>
<td>147</td>
<td>2.46 ± 0.46</td>
</tr>
<tr>
<td>N2</td>
<td>101</td>
<td>2.44 ± 0.50</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WD/MD</td>
<td>129</td>
<td>2.11 ± 0.21</td>
</tr>
<tr>
<td>PD</td>
<td>119</td>
<td>2.83 ± 0.67</td>
</tr>
</tbody>
</table>

Quantitative real-time PCR was used for detecting the expression level of miR-650 in 248 HCC tissues, mean, and SD values of miR-650 were shown as the table. Wilcoxon test was performed for correlation between miR-650 and clinicopathological factors: Gender of patients (male, n = 144; female, n = 104), age of patients (≤60, n = 79; >60, n = 169), tumor size (≤30 mm, n = 115; >30 mm, n = 133), tumor stage (I, n = 86; II, n = 114; III, n = 48), lymph node metastasis (N0/N1, n = 147; N2, n = 101), and differentiation (WD/MD, n = 129; PD, n = 119). P < 0.01.

**P-values were calculated by Wilcoxon test.***P < 0.05.

Fig. 2. Expression level of miR-650 is negative correlated with ING4 expression in tumor samples. A: Expression of miR-650 was evaluated in three normal tissues (N) and eight tumor tissues (T) by RT-PCR, as well as U6. B: The expression of ING4 was detected in these three normal tissues(N) and eight tumor tissues (T) by western blot. Positive expression of miR-650 was showed by “+” and negative expression was showed by “-” according gray value. C: The miR-650 and ING4 expression level were performed by quantitative real-time PCR in 122 HCC tissues. Scatter diagram of both two measured data was as shown (r = -0.2011, P = 0.0264).
miRNA population, was found to be frequently downregulated in HCCs and in all HCC-derived cell lines [12]. The let-7 family of miRNAs inhibits Bcl-xL expression and potentiates Sorafenib induced apoptosis [13]. Recently, miR-30d and miR-151, two frequently amplified miRNAs on chromosome 8q24, were found to be involved in HCC invasion and metastasis [14,15]. However, the role of miR-650 in HCC is poorly understood.

Previous studies showed that miR-650 was involved in lymphatic and distant metastasis in human gastric cancer and ectopic expression of miR-650 promotes tumorigenesis and proliferation of gastric cancer cells [7]. miR-650 was also found to be involved in expression inhibition of Cyclin-dependent kinase 1 though p16INK4a pathway [9]. Li et al. [16] found that down-regulation of NDRG2 gene expression in human colorectal cancer involved miR-650. In this study, we analyzed the expression of miR-650 in 120 paired HCC tissues and PCL tissues. The result showed that the expression of miR-650 was significantly enhanced in HCC tissues. Statistical analysis further indicated that miR-650 expression level is robust and higher both in younger patients (age ≤ 60) and poor differentiation. Our hypothesis in which miR-650 may be related to tumor malignancy, a total of 248 cases of HCC tissues were examined. We still found the expression level of miR-650 was significantly associated with age and poorly differentiated tumors. Moreover, miR-650 is critically related to tumor stage. Therefore, our data suggested that miR-650 may be involved in carcinogenesis of HCC.

For miRNAs, these approximately 19-22 nucleotide single stranded RNAs regulate genes by either inducing mRNA degradation or inhibiting translation. Zhang et al. [7] recently reported that miR-650 down-regulated ING4 by directly binding to the 3'UTR of ING4. ING4 is a member of the ING (inhibitor of growth) family, and has been regarded as a tumor suppressor gene. It is involved in apoptosis, cell cycle arrest, gene transcription, DNA repair, and other biological events [17]. Down-regulation of ING4 is associated with initiation and progression of lung cancer [18]. Reduced ING4 was also found in head and neck squamous cell carcinomas [19], gliomas, humangastric adenocarcinoma, [20] and correlates with tumor grade [21]. Deletion of ING4 locus has been found in breast cancer cells [22]. Moreover, decreased expression of ING4 was correlated with poor prognosis of HCC [23]. Based on this information, we detected ING4 expression and miR-650 level in 122 HCC specimens, and observed significant negative correlation between ING4 and miR-650 by statistical analysis. It is suggested that miR-
650 is involved in degradation process of ING4 in HCC; it may be a reason for down-regulated ING4 in lung cancer. It was also verified by further study about miR-650 role in normal liver cells. The Correlation coefficient $r_0$ is $-0.2011$ implied that miR-650 is not the only way for ING4 degradation, other mechanism may be involved in the process but have never been reported so far.

In summary, the differential expression of miR-650 in HCC patients of various age, tumor stages, degree of differentiation suggested that miR-650 may be critical to the pathogenesis of HCC. It offers a novel tool that has the potential to eventually influence the prevention, diagnosis, and therapy for HCC.

REFERENCES