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Original Article

MicroRNA-34b/c regulated by p53 is associated with unfavorable prognosis in patients with early hepatocellular carcinoma

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Abstract: Backgrounds: To identify the association between miR-34b/c expression levels and the p53 tumor suppressor in a Chinese cohort of patients with early hepatocellular carcinoma (EHCC) and explore the potential interrelation of these risk factors with the prognosis of EHCC. Methods: We retrospectively reviewed 80 patents with EHCC (14 female, 66 male) managed in our institution between 2007 and 2013. The expression of miR-34b/c and p53 were detected by real-time PCR and western blot. Prognostic factors were evaluated using Kaplan-Meier curves and Cox proportional hazards models. Results: For the entire cohort of 80 patients, the normalized real-time PCR results showed that p53 and miR-34b/c mRNAs were dysregulated in tumor tissues compared to the corresponding non-tumorous tissue samples. We next performed western blot and immunostaining to identify p53 expression levels in EHCC patients. Kaplan-Meier curves suggested that p53 and miR-34b/c had prognostic significance in this relatively selected cohort. We performed further multivariate Cox proportional hazards analysis combined the variables of p53 positive and low expression of miR-34b and miR-34c, respectively. After that we found that combined p53 positive and low expression of miR-34b (HR: 2.458, P = 0.003), p53 positive and low expression of miR-34c (HR: 2.212, P = 0.012) were independent prognostic factors of patients with EHCC. Conclusions: p53, miR-34b and miR-34c were dysregulated in the tumor tissues compared with corresponding noncancerous tissue samples. We also confirmed that combined p53 positive and low miR-34b/c were independent factors associated with unfavorable prognosis in patients with EHCC.

Keywords: Earlyhepatocellular carcinoma, p53, miR-34b/c, prognosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer worldwide and the second most frequent cause of cancer death [1]. Half of these cases and deaths were estimated to occur in China and the incidence of EHCC is still increasing year by year [2, 3]. Improved diagnostic technologies and more detailed physical examination of high risk people have increased the frequency of diagnosis of early hepatocellular carcinoma (EHCC).

Partial hepatectomy remains the standard curative treatment for EHCC, with a 5-year survival rate of over 50% for patients with EHCC [4, 5]. Unfortunately, postoperative recurrence rate vary from 40% to 70% at 5 years, which is still the main cause of death after treatment [6, 7]. Accurately prognostic prediction of tumor relapse is important to facilitate screening of high risk patients and for decision on adjuvant therapy.

MicroRNAs (miRNAs) are short, endogenous, non-coding regulatory RNAs with 22-24 nucleotides in length, which are upstream regulators of gene expression and contribute to cancer development and progression by acting as oncogenes or tumor suppressor genes [8-10], regulating tumor cell behaviors including proliferation, apoptosis, differentiation and metastasis in various cancer [11, 12]. Among the various miRNAs, the miR-34b/c gene, which localizes to chromosome 11q23.1 and belongs to the miR-34 family, shares a common primary transcript and acts as a potential tumor suppressor that is directly regulated by p53 [13,

Table 1. Patient and tumor characteristics (N = 80)

Variable	Median/ number	Range/ percent
Age in yr (median range)	50.23±10.2	31-72
Gender: Male/Female	66/14	82.5/17.5
HBsAg: Positive/Negative	62/18	77.5/22.5
HBeAg: Positive/Negative	29/51	36.25/63.75
TBL (μmol/l)	12.2±11.3	3.1-78.5
Albumin (g/dl)	40.3±5.2	24.2-52.1
ALT (U/L)	48.7.1±22.5	6.3-321.8
PT (S)	12.7±1.2	10.2-14.8
PLT (*10^9/L)	147±58	45-369
AFP ≤ 400/> 400 ng/ml	48/32	60/40
Blood transfusion: Yes/No	12/68	15/85
Edmondson-Steiner grade: I or II/III or IV	25/55	31.25/68.75
Cirrhosis: Yes/No	48/32	60/40
p53: Negative/Positive	47/33	58.75/42.25
MicroRNA-34b: High/Low	32/48	40/60
MicroRNA-34c: High/Low	37/43	46.25/53.75
Tumor size (all ≤ 5 cm)		
Size in cm (median, range)	2.9±1.2	0.5-5.0
Size: ≤ 2 cm/> 2	24/56	30/70
Microvascular invasion: Yes/No	25/55	31.25/68.75
Tumor Number: Multiple/solitary	20/60	25/75

14]. MiR-34b/c are involved in mediating cellular responses, such as cell cycle arrest [15], apoptosis, andmetabolic regulation [16].

As we know, p53 gene displays the highest correlation with various human types of cancer thus far. The past decade has witnessed three shifts in the understanding of the association between p53 and cancer, starting from p53 as a protein antigen to p53 as a cancer-associated gene, and finally, to p53 as a tumor-suppressor gene [17]. Interestingly, p53 not only regulates the expression of protein-coding genes but also regulates the maturation of miRNAs, lead to attenuation of miRNA processing activity [18, 19]. MiR-34b and miR-34c have been shown to bear p53 response elements in their 5' flanking regions and therefore are p53 transcriptional targets, involved in the regulation of cell cycle arrest, apoptosis and senescence [20]. with regard to EHCC, p53 dysregulation and miR-34b/c attenuation may play an important combined role in the pathogenesis of EHCC. In order to shed light on the role of p53 and these two miRNAs, in the present study, we analyzed the expression of these miR-34b/c and p53 in surgically resected EHCC patients and investigated their potential relationship with disease-free survival (DFS). Furthermore, we investigated the relationship of miR-34b and miR-34c with the dysregulation of p53 and the combined prognostic significance of these factors on patients with EHCC after curative hepatectomy.

Materials and methods

Patients and tissue samples

A total of 80 patients with early EHCC who underwent a curative liver resection at the Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, were included in this retrospective study. These patients were diagnosed as EHCC between May 1st 2007 and June 30th 2013. The inclusion criteria were patients with: (1) A preoperative ECOG criteria score of 0-1 [21]; (2) Child-Pugh class A; (3) No anti-cancer treatment before surgery; (4) Histologically proven EHCC in the resected specimen; (5) Tumors met the Milan criteria of a single tumor < 5 cm, or multiple tumors of < 3 in number, each < 3 cm [22, 23]; (6) No extrahepatic metastasis and tumor invasion into major portal/hepatic vein; (7) Complete resection of tumor according to the criteria that was previously reported [24]; (8) All patients were HCV negative. Patients who received liver resection with microscopic/macroscopic margin involvement by tumor (R1/R2 resection), and any preoperative mortality were excluded from this study.

The tissues were immediately frozen in liquid nitro-gen after surgical removal and stored at -80°C until use. None of the patients recruited in this study had undergone preoperative chemotherapy or radio-therapy. Tumor staging was determined according to the seventh edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer. The characteristics of patients were shown in **Table 1**. The study was approved by the Research Ethics Committee of Eastern Hepatobiliary Surgery Hospital. Informed consent was obtained from all patients.

RNA extraction and miRNA quantification

Total RNA was extracted from fresh frozen tissues using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. We typically extracted 2 μg to 9 μg of total RNA, and OD260/280 ratios typically ranged from 1.8 to 2.0, indicating high RNA purity. 10 ng of total RNA was used for each miRNA quantification. miRNA detection was performed run on the Eppendorf Mastercycler EP Gradient S (Eppendorf, Germany) using commercial assays (TaqMan microRNA assays; Applied Biosystems, Foster City, CA, USA) for miRNAs. Relative quantification was calculated using 2-ΔΔCt, where Ct is cycle threshold. Normalization was per-formed with universal small nuclear RNA U6 (RNU6B). Each sample was examined in triplicate, and the mean values were calculated. The ratio of mRNA levels in tumor versus non-tumorous samples of 0.5-fold was defined as under-expression of the gene, whereas a ratio of 2.0-fold was defined as over-expression.

Immunohistochemistry and evaluation of immunostaining

Immunohistochemical staining was performed with the Dako Envision Plus System (Dako, Carpinteria, CA) according to the manufacturer's instructions. The primary antibodies used was anti-p53 (Cell Signaling Technology Inc., Beverly, MA, 1:200) The tissue was evaluated as positive for p53 staining when there were more than 10% of tumor cells demonstrating cytoplasmic and/or nucleus immunoreaction deposits. The sections were scored with a four-tier scale: 0 = negative (0-10%), 1 = weak signal (10-20%), 2 = intermediate signal (20-50%) and 3 = strong signal (> 50%). 0 and 1 were defined as low, while 2 and 3 were defined as high. All sections were scored independently by two observers who did not have any prior knowledge of the clinicopathologic data. The concordance between scores from different sections of the same tumor was greater than 90%. All discrepancies in scoring were reviewed and a consensus was reached.

Western blotting analysis

Fresh surgical specimens were snap frozen in liquid nitrogen and stored in deep freezer. The normal tissues and the tumor were lysed in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) containing proteinase inhibitors (CalBiochem, San Diego, CA). The extracts were collected and centrifuged at 12,000×g for 5 min. The protein concentrations were determined using the BCA Protein Assay (Pierce) according to the manufacturer's instructions. The following antibodies were used: anti-p53 (Cell Signaling Technology Inc., Beverly, MA), we also used β-actin as a loading control.

Follow-up

Postoperative serum AFP and abdominal ultrasound were carried out in all patients monthly. Patients received abdominal contrast-enhanced CT scan or MRI once every 3 months in the first two years after surgery, and once every 6 months thereafter. Further investigations were carried out when clinically indicated or when tumor recurrence was suspected. Outcome definitions: Complete resection was defined as resection of all tumor sites on the basis of surgical findings and postsurgical images. OS was defined as the period from the date of surgery until death or last contact. Patients who did not experience an event were censored on the date of last contact. EFS was defined as the period from the date of surgery until an occurrence of event (progressive disease, death, diagnosis of a second malignant neoplasm) or last contact, whichever occurred first.

Statistical methods

Continuous variables were expressed as mean ± SD (standard deviation) and compared using a two-tailed unpaired Student's t test; categorical variables were compared using χ^2 or Fisher analysis. The cut-off of AFP level was defined by the receiver-operating characteristic (ROC) curve analysis [25]. Life-table estimates of survival time were calculated according to the Kaplan and Meier methodology [26]. The Greenwood formula was used for the standard deviation. A Cox proportional hazards regression approach [27] was chosen for the evaluation of EFS as the primary end-point. Potential prognostic variables were analyzed both univariately with one factor taken at a time, and then in a multivariate model combining all factors. Results were showed as hazard ratios (HR) and their 95% confidence intervals (CI) A HR > 1 indicated an elevated risk with respect

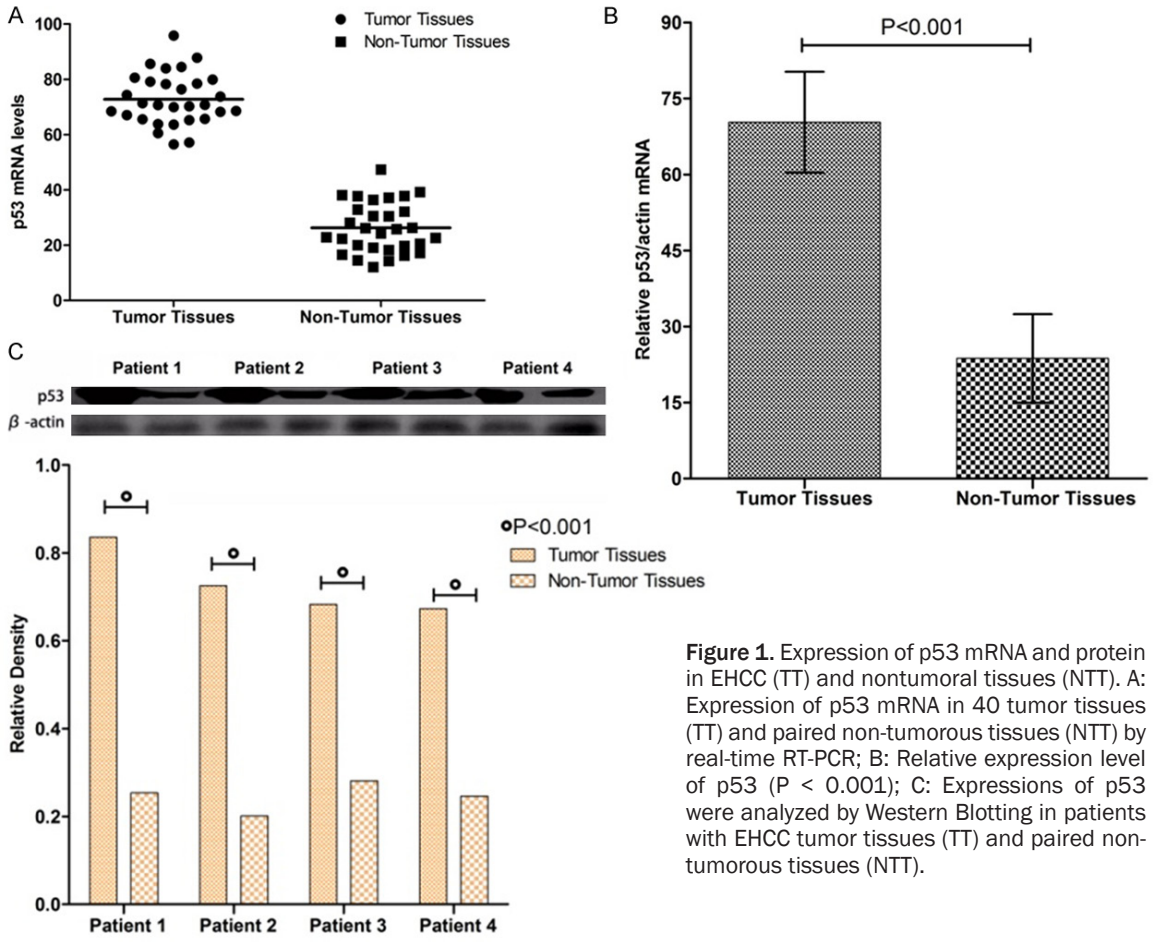

Figure 1. Expression of p53 mRNA and protein in EHCC (TT) and nontumoral tissues (NTT). A: Expression of p53 mRNA in 40 tumor tissues (TT) and paired non-tumorous tissues (NTT) by real-time RT-PCR; B: Relative expression level of p53 ($P < 0.001$); C: Expressions of p53 were analyzed by Western Blotting in patients with EHCC tumor tissues (TT) and paired non-tumorous tissues (NTT).

to the reference category. A confidence interval which did not include the value 1 indicated statistical significance at the 5% level. It should be noted that this was a retrospective evaluation and therefore statistical significance should be interpreted with caution. All statistical evaluations were carried out using SPSS software (Statistical Package for the Social Science, version 15.0, SPSS Inc, Chicago, IL). A value of $P < 0.05$ was considered to be statistically significant in all the analyses.

Results

Patients' characteristics

80 patients were recruited into this study. The median follow-up was 50.23±10.2 years (range 10-72 years). The baseline characteristics of patients at diagnosis were summarized in **Table 1**. Overall, the main gender was male (M:F = 4.71:1). most patients had HBsAg (77.5%). Most patients had no microvascular invasion (68.75%), and solitary tumor (75%). most patients had tumor > 2 cm (70%). The over-expression of p53, miR-34b, and miR-34c were 42.25%, 60% and 53.75%, respectively.

p53 mRNA and protein expression in normal and tumor tissues

The normalized real-time PCR results showed that p53 mRNA were over-expression in tumor tissues as compared with corresponding non-tumorous tissue samples (**Figure 1A**, **1B**). We then detected the protein expression of p53 by western blotting. We found increased expression level of p53 in 32 of 40 (80%) tumor tissues compared with their normal counterparts (**Figure 1C**). We next performed immunostaining in the 80 paired EHCC samples and found that 37 (42.25%) patients were identified as over-expression (**Figure 3A**, **3B**).

Expression of miR-34b and miR-34c wassignificantly down regulated in human EHCC tissues

The normalized real-time PCR results showed that miR-34b and miR-34c were deregulated in tumor tissues as compared with corresponding

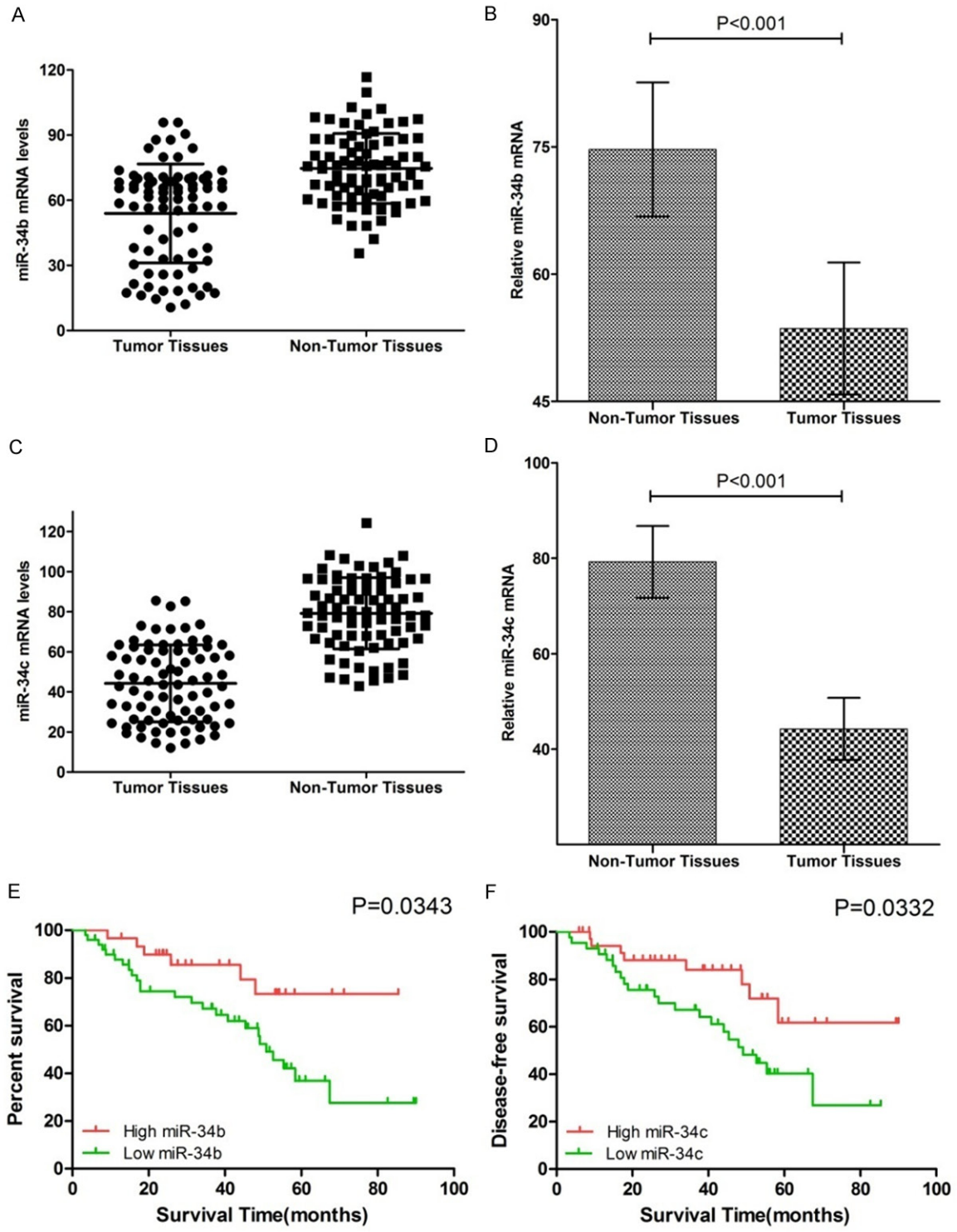

Figure 2. Expression and event-free survival analysis of miR-34b/c in patients with EHCC. A, B: Expression of miR-34b in tumor and normal tissue by relative RT-PCR quantitation; C, D: Expression of miR-34c in tumor and normal tissue by relative RT-PCR quantitation; E, F: Event-free survival analysis stratified by miR-34b/c.

non-cancerous tissue samples ($P < 0.01$, showed in **Figure 2A-D**). Both miR-34b and miR-34c were significantly down regulated in human EHCC tissues.

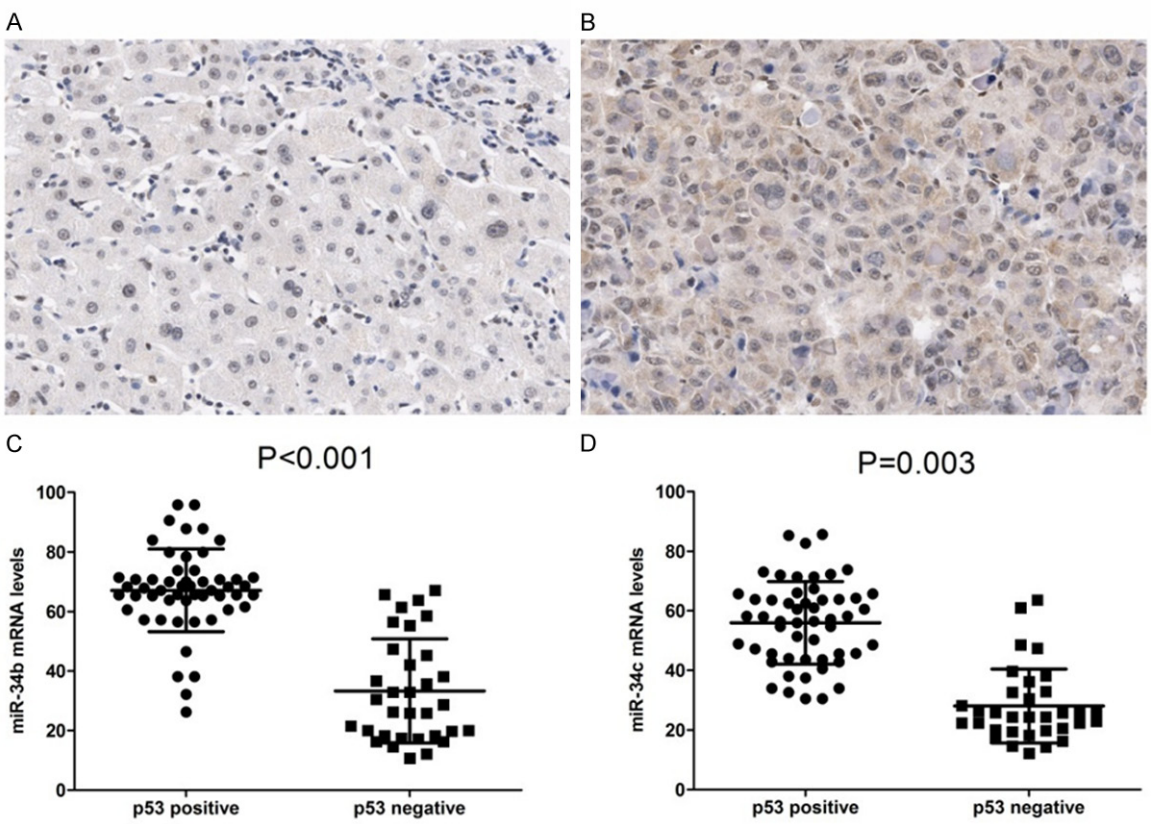

Figure 3. Representative immunohistochemical results of p53 expression negative (A) and p53 expression positive (B) were showed. MiR-34b (C) and miR-34c (D) expression levels were detected by relative RT-PCR quantitation according to the p53 expression.

Table 2. Descriptive survival statistics

Variables	1-year EFS Percent	3-year EFS Percent	5-year EFS Percent
Overall	91.1	73.8	47.6
Tumor size			
Size: ≤ 2 cm	98.5	92.1	78.6
Size: > 2 cm	86.7	68.3	31.4
Microvascular invasion			
Yes	90.4	83.3	62.5
No	63.1	50.8	36.1
Tumor Number			
Multiple	83.3	58.3	31.2
Solitary	95.4	80.2	56.9
p53			
Positive	84.2	62.1	29.6
Negative	95.7	81.2	64.2
MicroRNA-34b			
High expression	96.6	85.5	73.3
Low expression	85.5	67.2	36.8
MicroRNA-34c			
High expression	94.1	83.9	61.6
Low expression	90.6	67.2	40.3

Survival descriptions of miR-34b and miR-34c individually and in combination

For the entire cohort of 80 patients, the overall median survival was 78.6 months (95% CI: 68,3-86.7 months), and the 5-year EFS and OS rates were 47.6% and 63.5% (**Table 2**). Descriptive survival statistics and Kaplan-Meier curves suggested that tumor size, microvascular invasion, tumor number, p53 positive, low levels of miR-34b and miR-34c had prognostic significance in this relatively selected cohort. Low expression miR-34b was associated with a decreasing 5-year EFS rate from 73.3% to 36.8% (P = 0.0343, **Figure 2E**). Low expression miR-34c was associated with a decreasing 5-year EFS rate from 61.6% to 40.3% (P = 0.0332, **Figure 2F**). P53 positive was associated with a decreasing 5-year EFS rate from 64.2% to 29.6% (P = 0.007, **Figure 4A**).

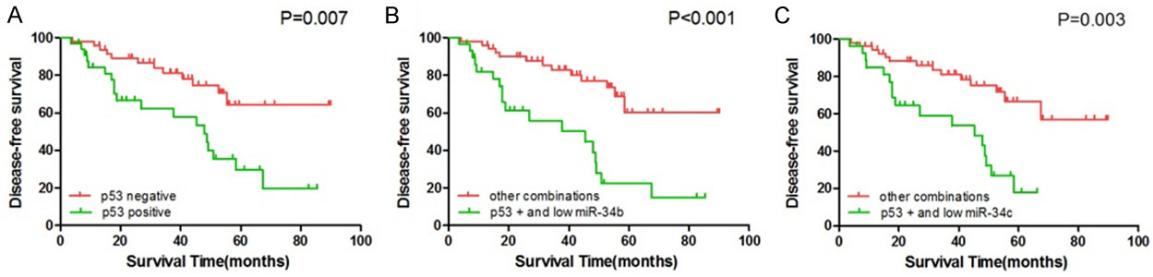

Figure 4. Kaplan-Meier curves for disease-free survival (DFS) in (A) patients with p53 positivity or negativity, patients with both p53 positivity and low miR-34b (B) and miR-34c (C) expression versus all other patients.

Table 3. Multivariable Cox proportional hazards analyses

Variables	Event-free Survival (EFS)			EFS (exclude miR-34c)			EFS (exclude miR-34b)		
	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
Tumor size (> 2 cm)	2.264	1.408-3.640	0.001	2.321	1.035-4.785	0.002	2.285	1.163-4.435	0.001
Microvascular invasion	1.955	1.105-3.461	0.006	2.637	1.775-4.075	< 0.001	2.348	1.452-4.836	< 0.001
Tumor Number	1.912	1.074-3.856	0.013	1.875	1.321-4.076	0.015	2.321	1.382-3.963	0.005
p53 positive	1.871	1.257-4.739	0.025	–	–	–	–	–	–
Combined p53 positive and low miR-34b	–	–	–	2.458	1.327-4.863	0.003	–	–	–
Combined p53 positive and low miR-34c	–	–	–	–	–	–	2.212	1.276-4.749	0.012

Abbreviations: CI, confidence interval.

Relationship of p53 positivity and miR-34b/c expression levels

In order to further explore the correlation of p53 and miR-34b/c expression levels affected on the EFS of patients with EHCC. We performed stratified analysis using immunohistochemical analysis. Immunohistochemical study revealed that there were 37 cases (42.25%) of p53 positivity in tumor tissues. Representative immunohistochemical results are shown in **Figure 3A**, **3B**. The normalized real-time PCR results from the 80 tumor samples showed that patients with p53 positivity and negativity express miR-b/c at different levels, respectively. MiR-34b and miR-34c expression in patients with p53 positivity was higher than patients with p53 negativity ($P < 0.001$ and $P = 0.003$, respectively; **Figure 3C**, **3D**).

Cox proportional hazard analysis

Cox proportional hazards models were then used to quantify the prognostic significance of risk factors after multivariable adjustment. A multivariable analysis was performed to assess the factors that demonstrated significant effects as in univariate analysis. After adjusting for competing risk factors, we identified that tumor size (> 2 cm), microvascular invasion, tumor number and p53 positive were associated with a worse prognosis in the multivariable adjusted analysis. MiR-34b and miR-34c expression levels showed no significant effect on the prognosis of patients with EHCC. We believed that some interaction or colinearity existed between p53 and miR-34b/c. Thus, we performed further multivariate Cox proportional hazards analysis combined the variables of p53 positive and low expression of miR-34b and miR-34c, respectively. After that we found that combined p53 positive and low expression of miR-34b (HR: 2.458, P = 0.003), p53 positive and low expression of miR-34c (HR: 2.212, P = 0.012) were independent prognostic factors of patients with EHCC (**Figure 4B**, **4C**; **Table 3**).

Discussion

With the implementation of various detection approaches, Early EHCC has increased significantly [28]. Generally, patients with EHCC had been considered as a cohort with relatively good prognosis after partial hepatectomy [29]. However, it remains controversial about the treatment modalities and the prognosis of patients with EHCC may vary since several risk factors influenced the EFS following partial hepatectomy [30]. The ability to accurately pre-

dict prognosis helps to look for early tumor recurrence, to select effective adjuvant treatment, and hopefully to improve survival [5, 31].

The tumor suppressor p53 gene is one of the most frequently mutated genes in human cancers including EHCC [32, 33]. MiRs are promising biomarkers and involved in regulating diverse biologic processes such as cell proliferation, apoptosis, adhesion, migration, invasion, and angiogenesis. Together with the protein-coding genes, several miRNAs also act as important components of the p53 signaling cascades and thereby contribute to tumor suppression, mediate and regulate the malignant characters of multiple tumors. MiR-34b and miR-34c, two members of the miR-34family, are the first miRNAs that have been found to be directly regulated by p53 [18], when ectopically expressed, miR-34 family display tumor suppressive activities in tumor biology [34]. Their expression may be induced by p53 in response to DNA damage or cell stress [35] as wellas regulated by DNA methylation. In this present study, our results showed significant differences in the expression levels of miR-34b and miR-34c between tumor and the corresponding adjacent tissues from surgically resected EHCC patients. MiR-34b and miR-34c expression were lower in tumor than in normal tissue, which were all consistent with previous reports. After further analysis, we found that miR-34b and miR-34c also associated with p53 expression. Patients with p53 positivity and negativity expressed miR-b/c at different levels. MiR-34b and miR-34c expression in patients with p53 positivity was higher than patients with p53 negativity. The underlying reason is still unclear and further evidence is needed.

With respect to the survival analysis of different risk factors affected on patients with EHCC, p53 positive, low expression of miR-34b and miR-34c were associated with unfavorable prognosis in patients with EHCC in univariate analysis of Cox regression model. To our surprise, only the variable of p53 positive showed statistical significance on the prognosis after multivariable adjustment. The potential reason was the interaction between miR-34b and miR-34c and the regulation of p53. Therefore, we then performed further analysis after combined the variables of p53 positive and low expression of miR-34b and miR-34c, respectively. After that we found that combined p53 positive and low expression of miR-34b, p53 positive and low expression of miR-34c was independent prognostic factors of patients with EHCC. These new findings provide a direction for our future study in predicting prognosis of patients with EHCC.

To the best of our knowledge, while there are many recognized prognostic and predictive markers for EHCC, including several clinicpathological characteristics and protein and gene signatures, the present study is the first to explore the potential implications for miRNAs regulated by p53 associated with EFS of patients with EHCC after curative hepatectomy. Meanwhile, the present study has several limitations. Firstly, this is a retrospective study and the prognostic factors we discussed are common factors including tumor size, microvascular invasion and tumor number; secondly, no further mechanism research been performed after the results of miRNAs regulated by p53 associated with EFS found. We would further explore the potential mechanism of miR-34s affecting on the prognosis of EHCC through the p53 tumor suppressor pathway.

In conclusion, we found that p53, miR-34b and miR-34c were dysregulated in tumor tissues compared with corresponding noncancerous tissue samples. We also confirmed that combined p53 positive and low miR-34b/c were independent factors associated with unfavorable prognosis in patients with EHCC.

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Disclosure of conflict of interest

None.

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