European Review for Medical and Pharmacological Sciences 2017; 21: 742-747

Expression of microRNA-122 and microRNA-22 in HBV-related liver cancer and the correlation with clinical features

D.-D. QIAO[1], J. YANG[2], X.-F. LEI[2], G.-L. MI[1], S.-L. LI[2], K. LI[2], C.-Q. XU[2], H.-L. YANG[2]

[1]Shandong Provincial Mental Health Center, Jinan, China
[2]Department of Gastroenterology, Shandong Provincial Qianfoshan Hospital, with Shandong University, Jinan, China

Dongdong Qiao and *Jing Yang* contributed equally to this work

Abstract. – OBJECTIVE: MicroRNAs (miR) participate in cell proliferation, apoptosis and transformation, as they can regulate gene expression and intracellular signal transduction for various physiological processes. MiR-122 and miR-22 are known to be related with occurrence and progression of hepatitis B virus (HBV)-related hepatocellular cancer (HCC). This study recruited HBV-related HCC patients, whose expression levels of miR-122 and miR-22 were determined to analyze the correlation with clinical and pathological indexes.

PATIENTS AND METHODS: HBV-related HCC patients were enrolled, in parallel with patients suffering from benign liver disease and non-HBV-related HCC. Real-time PCR was employed to measure miR-122 and miR-22 expression levels.

RESULTS: The relative expression levels of miR-122 and miR-22 in HBV-related HCC patients were 1.26 ± 2.73 and 5.49 ± 3.91, respectively, which were significantly lower than that in benign liver disease or non-HBV-related HCC patients ($p < 0.05$). No significant difference of serum miR-122 or miR-22 levels was found between benign liver disease and non-HBV-related HCC patients ($p > 0.05$). The miR-122 and miR-22 levels were negatively correlated with tumor size, lymph node metastasis, TNM stage, pathological type, differentiation grade, liver cirrhosis, AFP and HBV DNA, all of which were independent risk factors ($p < 0.05$).

CONCLUSIONS: MiR-122 and miR-22 were downregulated in HBV-related HCC patients, and were related with tumor size, lymph node metastasis, TNM stage, pathological type, differentiation grade, liver cirrhosis, AFP and HBV DNA.

Key Words:
HBV-related hepatocellular carcinoma, microRNA-122, microRNA-22, Clinical pathological features.

Introduction

Primary hepatocellular carcinoma (HCC) is the fifth popular cancer worldwide, with high invasiveness and high mortality[1]. About 350 million people were infected with hepatitis B virus (HBV), of which there were about 120 million infected people in China. Patients often develop liver cirrhosis, or even primary liver cancer. Each year there are about 1 million people died from HBV-related liver disease[2,3]. microRNA (miR) is a type of single-stranded small molecule RNA, with 18~25 nucleic acids. It can regulate the expression level of target genes via binding onto 3'-untranslated region (3'-UTR)[4]. Previous studies[5,6] indicated a large difference of specific miR expression in various malignant tumors including breast cancer, liver cancer, pulmonary carcinoma, gastric cancer and ovarian carcinoma. Among those, miR-122 and miR-22 can specifically bind with HBV RNA, and are thus related to occurrence and progression of HBV-related HCC. This work recruited such patients who received surgeries in our hospital, to detect the expression of miR-122 and miR-22 levels in all patients before and after surgery, and analyze its correlation with TNM stage, and lymph node metastasis of HBV-related HCC.

Patients and Methods

General Information

A total of 40 HBV-related HCC patients who received surgical resection from January 2014 to January 2014 in Shandong Qianfoshan

Corresponding Author: Hongli Yang, MD; e-mail: hongliyang588@sina.com

Hospital Affiliated with Shandong University were enrolled as the disease group. All patients were diagnosed as HCC by pathological examination, and did not receive chemo-/radio-therapy. All patients were detected positive for serum HbsAg or HBV cccDNA. Typical tumor lesion samples and adjacent tissues (15 cm from the tumor lesion edge, without cancer cells by pathological examination) were collected. In all patients, there were 20 males and 20 females, aging between 31 and 81 years old (average = 51.23 ± 2.63 years). Another cohort of 40 benign liver disease patients (including 16 cases of hepatic focal nodular hyperplasia, 13 cases of hepatic hemangioma and 11 cases of hepatic trauma, aging between 34 and 70 years, average age = 49.78 ± 3.87 years) was also recruited. The control group included 40 non-HBV-related HCC patients (20 males and 20 females, aging between 34 and 70 years, average age = 48.36 ± 4.12 years). No significant difference existed regarding sex or age of individuals among these three groups ($p > 0.05$). Venous blood sample was collected and frozen. 100 mg tissue samples were collected immediately after resection and were kept at -80°C.

The experimental protocol has been pre-approved by the Ethical Committee of Shandong Qianfoshan Hospital Affiliated with Shandong University and written consents have been obtained from all patients and healthy volunteers.

Inclusive Criteria

All patients were diagnosed with pathology examination, without connective tissue disease, immune disorder, or received chemo-/radio-/immune-/frozen-/laser- treatment before the surgery.

Exclusive Criteria

Patients were diagnosed with dysfunction of major organs including heart, liver or kidney, with other malignant tumors, chronic/acute infectious disease or mental/psychological disease that were incompliance with treatment.

Equipment and Reagent

Real-time PCR kit for miR-122 and miR-22 (Takara, Shuzo, Japan); Roche 480 qPCR (Roche, Indianapolis, IN, USA); Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA); RNase-free H_2O (Sangon, Shanghai, China); NanoDrop UV spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

Primer Design

All primers were synthesized by Sangon (Sangon, Shanghai, China) as shown in Table I.

Real-time PCR for Serum and Tissue miR-122 and miR-22 Expression

Fasted blood samples were collected from disease, benign liver disease and control group patients and were placed into EDTA-containing tubes. At 1500 g, 4°C centrifugation for 20 min, serum was collected and kept at -20°C. For tissue samples, fresh tissues samples were collected during the surgery and were frozen in liquid nitrogen, and were kept at -80°C for further use. TRIzol reagent was employed to extract total RNA from sterile RNAase-free environment following the manual instruction. 1 μg total RNA was used to synthesize cDNA by reverse transcription using oligoT18. Quantitative PCR was used to determine the relative expression of target genes under the following conditions: 95°C pre-denature for 2 min, followed by 40 cycles each containing 94°C denature for 1 min, 60°C annealing 1 min and 72°C elongation 1 min. At the end of the last cycle, the sample was kept from 55°C to 95°C gradient (30 s for every 0.5°C). Curve analysis was then performed. U6 was set as an internal reference.

Statistical Analysis

SPSS17.0 statistical software (IBM, Armonk, NY, USA) was used for the statistical analysis. Data were presented as mean±standard deviation (SD). Measurement data were compared by student *t*-test. One-way analysis of variance (ANOVA) was performed to compare means

Table I. Primers sequence.

Target gene	Forward primer	Reverse primer
miR-122	5'-GCGAA AGCAT TTGCC AAGAA -3'	5'-CATCA CAGAC CTGTT ATTGC-3'
miR-22	5'-AAATC ACCAC CTTCA CAGCC-3'	5'-GTTGT AATGG TTCTC CTCCA GC-3'
U6	5'-AGCGG GAAAT CGTGC GTGAC A-3'	5'-GTGGA CTTGG GAGAG GACTG G-3'

Table II. Serum miR-122 and miR-22 expression in patients.

Target gene	HBV-related HCC	Benign liver disease	Control
miR-122	1.26 ± 2.73*,#	10.54 ± 0.32	11.35 ± 0.31
miR-22	5.49 ± 3.91*,#	10.91 ± 0.44	11.73 ± 0.43

Note: *, $p < 0.05$ compared to benign disease group; #, $p < 0.05$ compared to control group.

across groups. χ^2 test was used to compare enumeration ratios. Statistical significance was identified when $p < 0.05$.

Results

Serum miR-122 and miR-22 Expression in Patients

RT-qPCR was used to test serum miR-122 and miR-22 expression levels in all patients. Significant lower expression levels of miR-122 and miR-22 in HBV-related HCC patients (1.26 ± 2.73 and 5.49±3.91, respectively) were found, compared with that in benign liver disease group or control group ($p < 0.05$). No significant difference of levels between benign disease and control groups was found ($p > 0.05$, Table II).

MiR-122 and miR-22 Expression in Lver Cancer Tissues and Adjacent Tissues

We further tested miR-122 and miR-22 expression levels in both tumor tissues and adjacent tissues from HBV-related HCC patients. Results showed that the relative expression level of miR-122 and miR-22 in tumor tissues was 0.183 ± 0.025 and 0.149 ± 0.068, respectively. These levels were also significantly lowered than those in tumor adjacent tissues (0.698±0.024 and 0.712 ± 0.032, respectively, $p < 0.05$, Figure 1).

MiR-122 and miR-22 Expression and Clinical/Pathological Features

We further analyzed the correlation between miR-122/miR-22 expression in tumor tissues and HBV-related HCC patients' profiles, including sex, age, tumor lesion size, lymph node metastasis, TNM stage, pathological type, differentiation grade, liver cirrhosis, AFP and HBV DNA. Results showed the correlation between miR-122 or miR-22 expression and characteristics such as tumor size, lymph node metastasis, TNM stage, pathological type, differentiation grade, liver cirrhosis, AFP and HBV DNA. Those patients, with tumor size larger than 5 cm, with lymph node metastasis, late TNM stage, HCC, lower differentiation grade, liver cirrhosis history, AFP higher than 20 ng/ml or HBV DNA high-expression, exhibited significantly lower miR-122 or miR-22 expression levels ($p < 0.05$). No correlation existed between miR-122 or miR-22 expression levels and sex or age ($p > 0.05$, Table III).

Multi-Variant Analysis Between miR-122/miR-22 and Clinical/Pathological Features

Logistic multi-variant analysis was further performed for the evaluation of miR-122/miR-22 levels with tumor size, lymph node metastasis, TNM stage, pathological type, differentiation grade, liver cirrhosis, AFP and HBV DNA. Results demonstrated that tumor size, lymph node metastasis, TNM stage, pathological type, differentiation grade, liver cirrhosis and AFP, were all independent risk factors (Table IV).

Discussion

HBV severely affects people and brings a socio-economic burden in China. Chronic HBV infection leads to liver cirrhosis or primary HCC

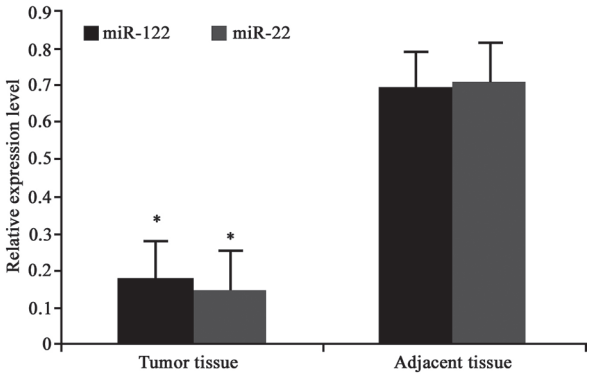

Figure 1. Relative expression levels of miR-122 and miR-22 in tumor and adjacent tissues from HBV-related HCC patients. *, $p < 0.05$ compared to tumor adjacent tissues.

Table III. Relationship between miR-122 and miR-22 expression levels and clinical/pathological features of HBV-related HCC patients.

Parameter	N	miR-122	*p*-value	miR-22	*p*-value
1. Sex			0.176		0.135
Male	20	0.676 ± 0.412		0.112 ± 0.432	
Female	20	0.683 ± 0.315		0.103 ± 0.525	> 0.05
2. Age	40		0.257		0.187
> 50 years	19	0.628 ± 0.415		0.141 ± 0.201	
≤ 50 years	21	0.645 ± 0.21		0.428 ± 0.103	
3. Tumor size	40		0.026		0.019
≤ 5 cm	22	2.221 ± 0.872		2.018 ± 0.714	
> 5 cm	18	0.988 ± 0.527		0.875 ± 0.312	
4. Lymph node metastasis	40		0.021		0.011
No	21	2.221 ± 0.872		2.018 ± 0.714	
Yes	19	0.912 ± 0.314		0.804 ± 0.203	
5. TNM stage	40		0.044		0.041
Stage I	14	2.213 ± 0.662		2.099 ± 0.508	
Stage II	17	1.461 ± 0.441		1.363 ± 0.427	
Stage III	9	0.703 ± 0.113		0.507 ± 0.103	
6. Pathological type	40		0.024		0.021
HCC	18	1.014 ± 0.213		1.013 ± 0.122	
CCC	14	1.438 ± 0.418		1.267 ± 0.312	
Mixed	8	2.234 ± 0.726		2.373 ± 0.721	
7. Differentiation grade	40		0.022		0.016
High	19	3.201 ± 0.801		2.689 ± 0.572	
Moderate	10	1.238 ± 0.617		1.459 ± 0.242	
Low	11	0.287 ± 0.512		0.201 ± 0.103	
8. Liver cirrhosis	40		0.034		0.025
No	21	2.127 ± 0.532		2.005 ± 0.414	
Yes	19	0.964 ± 0.227		0.811 ± 0.212	
9. AFP	40				
< 20 ng/ml	16	2.116 ± 0.601		2.001 ± 0.592	
20 ng/ml	24	0.745 ± 0.127		0.524 ± 0.101	
10. HBV DNA					
High		2.097 ± 0.483		2.113 ± 0.402	
Low		0.755 ± 0.186		0.662 ± 0.233	

Note: HCC, hepatocellular carcinoma; CCC, cholangiocarcinoma.

or other severe liver diseases[7]. MiR is widely distributed in various animal and plant cells. It is a type of non-coding small molecule RNAs, which are characterized as about 22 nucleotides, highly conserved sequence[8]. A previous study[9] found the involvement of miR in the regulation of cell differentiation, proliferation, growth, migration and apoptosis, as it can regulate 1/3 of

Table IV. Multi-variant analysis between miR-122/miR-22 and clinical/pathological features.

Index	microRNA22			microRNA22		
	Regression coefficient	*p*-value	Relative risk	Regression coefficient	*p*-value	Relative risk
Tumor size	0.724	0.003	2.125	0.801	0.004	2.228
Lymph node metastasis	1.132	0.002	2.027	1.275	0.001	2.852
TNM grade	1.007	0.001	2.004	1.018	0.001	2.812
Pathological type	1.113	0.002	2.892	1.004	0.001	2.730
Differentiation grade	0.748	0.002	2.156	0.952	0.003	2.376
Liver cirrhosis	1.022	0.001	2.313	1.001	0.002	2.468
AFP	1.005	0.001	2.247	1.008	0.002	2.502
HBV DNA	1.153	0.002	2.034	1.203	0.001	2.104

human genes, making it an important regulatory factor. MiR also participates in various viral infection processes. Previous studies[10,11] indicated that certain viruses could self-synthesize viral miR, and modify miR expression in host cells, to regulate viral replication even in a chronic infection way, thus exerting critical roles for viral pathogenicity. In this study, serum miR-122 and miR-22 levels were tested in HBV-related HCC patients, benign liver disease patients and non-HBV-related HCC patients. Our data demonstrated the critical role of miR-122 and miR-22 during the HBV infection owing to the down-regulation of miR-122 and miR-22 in HBV-related HCC, which was consistent with the previous finding by Zhang et al[12]. miR-122 exerted as an important marker targeting liver injury to different extents in serum of chronic HBV infection patients, with high sensitivity and specificity. Also, miR-122 can bind with highly conserved sequence of HBV RNA virus, and negatively regulate the expression and replication of viral genes. Besides, the deficient expression of miR-122 significantly enhanced replication potency of HBV via cell cycle protein G to mediate P53 activity[13]. Tan et al[14] indicated that a total of 8 miR molecules including miR-122 can predict the prognosis of HBV-related HCC, manifesting a promising predictors. Basic study in miR-122 knockout mice found severe abnormal metabolism of blood lipids, and further development to fatty liver or liver cancer. Exogenous introduction of miR-122 rendered the blockage of further development of mouse HCC[15]. To further study the correlation between miR-122/miR-22 expression and clinical/pathological feature of HBV-related HCC, we analyzed the relationship between miR-122 or miR-22 expression and tumor size, lymph node metastasis, TNM stage, pathological type, differentiation grade, liver cirrhosis, AFP and HBV DNA and showed that tumor size, lymph node metastasis, TNM stage, pathological type, differentiation grade, liver cirrhosis and AFP were independent risk factors. Of note, Chen et al[16] performed an *in vitro* study and found that miR-122 could induce the reduction of HBV gene expression. Wang et al[17] found that for those patients with HBV chronic infection, viral expression and replication were facilitated to certain extents via down-regulating host restrictive small RNA molecule and miR-122-cycline G1/p53-viral enhancer pathway. A current study has confirmed the close correlation between cyclin G1 and gene instability. Once cyclin G1 presented abnormal expression, an increasing amount of body cells were arrested in G1 phase, exerting certain regulatory functions to P53-dependent cell division pathway[18-20]. MiR-122, as a liver-specific small RNA, is abundant in liver. Both *in vivo* and *in vitro* studies demonstrated the down-regulation of miR-122 in HBV-related HCC tumor tissues, probably related with tumor's differentiation grade, tumor size, metastasis and prognosis[21,22]. Down-regulation of miR-122 inside body elevates the risk of HCC, whilst the abundant expression of miR-122 may have certain inhibitory effects on proliferation and growth of malignant tumor cells, as consistent with our study.

Conclusions

The down-regulation of miR-122 and miR-22 probably facilitates the recurrence and metastasis of HBV-related HCC and affects patient prognosis, although detailed mechanism requires further investigation.

Acknowledgements

This work was supported by the Technology Development Program of Shandong Province(NO.2014GGB1468), and the Medical and Health Technology Development Program of Shandong Province(NO.2014ws0366).

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Zhang ZZ, Liu X, Wang DQ, Teng MK, Niu LW, Huang AL, Liang Z. Hepatitis B virus and hepatocellular carcinoma at the miRNA level. World J Gastroenterol 2011; 17: 3353-3358.
2) Hsu SH, Wang B, Kota J, Yu J, Costinean S, Kutay H, Yu L, Bai S, La Perle K, Chivukula RR, Mao H, Wei M, Clark KR, Mendell JR, Caligiuri MA, Jacob ST, Mendell JT, Ghoshal K. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. J Clin Invest 2012; 122: 2871-2883.
3) Wang G, Dong X, Tian W, Lu Y, Hu J, Liu Y, Yuchi J, Wu X. Evaluation of miR-122-regulated suicide gene therapy for hepatocellular carcinoma in an orthotopic mouse model. Chin J Cancer Res 2013; 25: 646-655.

4) Skalsky RL, Cullen BR. Viruses, microRNAs, and host interactions. Annu Rev Microbiol 2010; 64:123-141.

5) Starkey Lewis PJ, Dear J, Platt V, Simpson KJ, Craig DG, Antoine DJ, French NS, Dhaun N, Webb DJ, Costello EM, Neoptolemos JP, Moggs J, Goldring CE, Park BK. Circulating microRNAs as potential markers of human drug-induced liver injury. Hepatology 2011; 54: 1767-1776.

6) Jopling C. Liver-specific microRNA-122: biogenesis and function. RNA Biol 2012; 9: 137-142.

7) Dienstag JL. Hepatitis B virus infection. N Engl J Med 2008; 359: 1486-1500.

8) Xu X, Fan Z, Kang L, Han J, Jiang C, Zheng X, Zhu Z, Jiao H, Lin J, Jiang K, Ding L, Zhang H, Cheng L, Fu H, Song Y, Jiang Y, Liu J, Wang R, Du N, Ye Q. Hepatitis B virus X protein represses miRNA-148a to enhance tumorigenesis. J Clin Invest 2013; 123: 630-645.

9) Hoffmann TW, Duverlie G, Bengrine A. MicroRNAs and hepatitis C virus: toward the end of miR-122 supremacy. Virol J 2012; 9:109.

10) Arataki K, Hayes CN, Akamatsu S, Akiyama R, Abe H, Tsuge M, Miki D, Ochi H, Hiraga N, Imamura M, Takahashi S, Aikata H, Kawaoka T, Kawakami H, Ohishi W, Chayama K. Circulating microRNA-22 correlates with microRNA-122 and represents viral replication and liver injury in patients with chronic hepatitis B. J Med Virol 2013; 85: 789-798.

11) Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem 2010; 285: 17442-17452.

12) Zhang Y, Jia Y, Zheng R, Guo Y, Wang Y, Guo H, Fei M, Sun S. Plasma microRNA-122 as a biomarker for viral-, alcohol-, and chemical-related hepatic diseases. Clin Chem 2010; 56: 1830-1838.

13) Zhu L, Chen Z, Chen JZ, Wang J, Hu ZR, Chen LW, Liu RH, Hu MJ, Zhu HH. [Effects of miR-122 on expression of hepatitis B virus proteins]. Zhejiang Da Xue Xue Bao Yi Xue Ban 2011; 40: 593-597.

14) Kwak MS, Lee DH, Cho Y, Cho EJ, Lee JH, Yu SJ, Yoon JH, Lee HS, Kim CY, Cheong JY, Cho SW, Shin HD, Kim YJ. Association of polymorphism in pri-microRNAs-371-372-373 with the occurrence of hepatocellular carcinoma in hepatitis B virus infected patients. PLos One 2012; 7: e41983.

15) Tsai WC, Hsu SD, Hsu CS, Lai TC, Chen SJ, Shen R, Huang Y, Chen HC, Lee CH, Tsai TF, Hsu MT, Wu JC, Huang HD, Shiao MS, Hsiao M, Tsou AP. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. J Clin Invest 2012; 122: 2884-2897.

16) Chen S, Ni M, Yu B, Lv T, Lu M, Gong F. Construction and identification of a human liver specific microRNA eukaryotic expression vector. Cell Mol Immunol 2007; 4: 473-477.

17) Wang S, Qiu L, Yan X, Jin W, Wang Y, Chen L, Wu E, Ye X, Gao GF, Wang F, Chen Y, Duan Z, Meng S. Loss of microRNA 122 expression in patients with hepatitis B enhances hepatitis B virus replication through cyclin G(1)-modulated P53 activity. Hepatology 2012; 55: 730-741.

18) Fornari F, Gramantieri L, Giovannini C, Veronese A, Ferracin M, Sabbioni S, Calin GA, Grazi GL, Croce CM, Tavolari S, Chieco P, Negrini M, Bolondi L. MiR-122/cyclin G1 interaction modulates p53 activity and affects doxorubicin sensitivity of human hepatocarcinoma cells. Cancer Res 2009; 69: 5761-5767.

19) Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu Cg, Calin Ga, Giovannini C, Ferrazzi E, Grazi GL, Croce CM, Bolondi L, Negrini M. Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. Cancer Res 2007; 67: 6092-6099.

20) Hou W, Bukong TN, Kodys K, Szabo G. Alcohol facilitates HCV RNA replication via up-regulation of miR-122 expression and inhibition of cyclin G1 in human hepatoma cells. Alcohol Clin Exp Res 2013; 37: 599-608.

21) Waidmann O, Koberle V, Brunner F, Zeuzem S, Piiper A, Kronenberger B. Serum microRNA-122 predicts survival in patients with liver cirrhosis. PLos One 2012; 7: e45652.

22) Nakao K, Miyaaki H, Ichikawa T. Antitumor function of microRNA-122 against hepatocellular carcinoma. J Gastroenterol 2014; 49: 589-593.