

Expression and Clinical Values of Serum miR-155 and miR-224 in Chinese Patients with HCV Infection

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Background: To investigate the expression of serum miR-155 and miR-224 among patients with hepatitis C virus (HCV) infection and analyze their clinical values.

Methods: A total of 116 patients suffering from HCV infection admitted to our hospital and 70 healthy subjects were selected. According to the diagnostic results, patients with HCV infection were divided into 48 cases of chronic hepatitis C (CHC), 43 cases of liver cirrhosis and 25 cases of hepatocellular carcinoma (HCC). The expression signature for miR-155 and miR-224 was detected in serum samples. ROC curve and Pearson correlation test were conducted to investigate their diagnostic value and correlation.

Results: The expression extent for serum miR-155 and miR-224 increased along with the increase of malignancy (all $P < 0.05$). According to ROC curve, the area under the curve (0.918, 95% CI: 0.856–0.974) of miR-155 and miR-224 combined in the diagnosis of HCC was the largest, and its sensitivity and specificity were 93.0% and 86.2%. There is a positive relationship for expression level between miR-155 and miR-224 in CHC and HCC group (all $P < 0.001$).

Conclusion: miR-155 and miR-224 are remarkably increased in patients suffering from HCV infection. The combination of miR-155 and miR-224 has a good diagnostic value for HCC caused by HCV infection.

Keywords: hepatitis C, hepatocellular carcinoma, miR-155, miR-224

Introduction

As a severe health issue across the world, hepatitis C virus (HCV) infection is expected to affect 1% of the global population, and the majority is not aware until the growth of advanced liver disease.¹ Nowadays, 400,000 people die of HCV infection each year. It is predicted that by 2040, more than 800,000 cases are expected to die from HCV-associated cirrhosis or HCC.^{2,3} HCV can trigger chronic hepatitis C (CHC), which is also one of the main causes of HCC. In China, chronic HCV infection is considered to be a very important factor for cirrhosis, chronic hepatitis and HCC. According to epidemiological studies conducted in the mid-1990s reported a national prevalence of 3.2%,⁴ but other reports suggest a range of 0.29% to 9.6%.⁴ The incidence of cirrhosis is 5% to 25% after 25 to 30 years of HCV infection,⁵ liver decompensation is 30% after 10 years of HCV-associated cirrhosis,⁵ and hepatocellular carcinoma is 1% to 3% per year.^{5,6} Chronic HCV infection is one of the most common risk factors for HCC. It is estimated that there are about 10 million HCV patients in China. Chronic hepatitis mainly causes extensive fibrosis and HCC through chronic inflammation, cell necrosis and regeneration.

There is currently no vaccine to prevent HCV infection. The combination of long-acting pegylated interferon type I (PEGIFN- α) and ribavirin (RBV) is the optimal treatment for hepatitis C in recent 10 years, but more than 50% of the patients with type I HCV still do not respond to this combination of antiviral therapy. At the same time, there are obvious toxic and side effects (such as influenza-like symptoms, anemia and depression, etc), and the treatment course is long, usually up to 1 year, so the clinical application of this therapy is limited. According to the current situation, antiviral therapy for chronic HCV infection has entered the era of direct antiviral drug pangenotype. A generic interferon-free regimen is preferred, which achieves a sustained virological response of more than 90% in HCV-infected patients with known major genotypes and major subtypes, and is consistent in multiple populations with different clinical characteristics and less drug interactions. Ribavirin (RBV) is not required in combination with interferon (IFN), except in a few specific populations, such as decompensated cirrhosis and DAAs failure. Therefore, the use of a pan-genotype regimen reduces pretreatment testing and in-treatment monitoring and is more suitable for the treatment and management of patients with chronic HCV infection at the primary level. Therefore, finding a reliable biomarker for the diagnosis of HCV-associated disease is of great importance.

MicroRNA (miRNA), is a cluster of about 21–25 nucleotides, which regulates gene expression through targeting mRNA. miRNA is small, conserved, noncoding and endogenous. miRNA is involved in a variety of biological and pathological processes such as cell development, signal transduction, cell growth, proliferation, movement and apoptosis.^{7,8} As a small-molecule RNA, is abnormally expressed in patients suffering from hepatitis C, participating in the occurrence and development of hepatitis C as a hidden target for hepatitis C treatment.^{9,10} Multiple miRNAs are dysregulating and have been recognized for being potential and promising biomarkers in HCC diagnosis and staging,^{11,12} including miR-30b,¹³ miR-485,¹⁴ miR-545,¹⁵ miR-30a-5p,¹⁶ miR-214,¹⁷ miR-497-5p¹⁸ and miR-9-5p.¹⁹ According to current researches, miR-155 and miR-224 participate in mediating hepatocyte immune response, exert a significant effect on the progression and metastasis of HCC, and are expected to become potential markers for the diagnosis of HCC.^{20,21} This study analyzed the expression of miR-155 and miR-224 among patients suffering from HCV-infected liver disease, so as to explore their diagnostic values for HCC and to provide guidance for diagnosing and treating HCC.

Materials and Methods

Research Objects

In total, 116 patients with HCV infection receiving treatment in our hospital from May 1, 2020 to May 31, 2021 were collected as the research objects, including 62 males and 54 females, with an average of (47.50 \pm 9.82) years. These patients were fallen into three groups including 48 cases of chronic hepatitis C, 43 cases of liver cirrhosis and 25 cases of HCC. The 116 patients were infected with HCV, including 76 cases of type 1B, 36 cases of type 2A, 3 cases of type 3A, and 1 case of type 3B. TNM stages of 25 HCC patients included stage I–II in 12 cases and stage III–IV in 13 cases. In this study, direct antiviral drugs were used to treat patients infected with HCV in accordance with the latest WHO guidelines for screening and prevention of chronic hepatitis C released in 2016. The antiviral drugs were mainly sofosbuvir combined with daclatasvir, and oral antiviral drugs of GI II and GI III. Another 70 cases undergoing physical examination were enrolled as controls including 39 males and 31 females, with an average of (48.73 \pm 8.95) years.

Inclusion and Exclusion Criteria

Inclusion criteria were as follows: 1) diagnosis of CHC refers to the criteria in the guidelines for preventing and treating hepatitis C²²; 2) HCV RNA was quantitatively positive in liver tissue. Exclusion criteria were as follows: 1) patients with liver disease induced by drug, fatty liver disease and hereditary metabolic liver disease; 2) serological examination combined with other types of hepatitis virus infection, EB virus, cytomegalovirus, HIV-infected persons, malignant tumors and immune diseases; 3) patients with severe heart, brain and kidney diseases; 4) HCV-infected persons who are receiving or have previously received interferon or interferon combined with ribavirin antiviral therapy.

Detection Indexes

The biochemical data measured and how they were done we consult the corresponding references.^{23,24} HCV-RNA detection: all subjects used SLAM96P fluorescence quantitative PCR instrument and reagent provided by Shanghai Hongshi Company to

detect HCV-RNA before treatment. And we quantified produced RNA using A260/A280 ratio.^{25–27} Detection of miR-155 and miR-224: miR-155 and miR-224 were detected by SLAM96P fluorescence quantitative PCR (Thermo Scientific, Waltham, Massachusetts, USA). The kit of RNA extraction: Invitrogen TRIzol (Thermo Scientific, Waltham, Massachusetts, USA). The total PCR system was 20 mL: 10 mL TaqMan general mixture solution (2×), 1 mL primer and probe Mix (20 ×), 1.33 mL reverse transcribed DNA, 7.67 mL double distilled water. The detailed experimental protocols and methods for total RNA extraction, cDNA synthesis, qRT-PCR and biochemical tests can be consulted in the literature by Chen et al.^{28,29} Forward primer: 5'-GCCGCTTAATGCTAATCGTG-3', reverse primer: 5'-CAGTGCTGGGTCCGACTGA-3' for miR-155 gene, forward primer: 5'-CAAGUCACUAGUGGUUCCGUU-3', reverse primer: 5'-TCCACCACTGTCCCGACT-3' for miR-224 gene and U6 gene was used as an internal control using the forward primer: 5'-CTCGCTTCGGCAGCACA-3' and reverse primer: 5'-CAAGTCACTAGTGGTTCCGTTTA-3'. We used the $2^{-\Delta\Delta Ct}$ calculation formula to determine the relative expression level as follows: $\Delta Ct = Ct$ value of target gene (miR-155 or miR-224) - Ct value of U6.^{30–33}

Statistical Methods

The statistical analyses were conducted through SPSS20.0 software. $\bar{x} \pm S$ was employed to represent measurement data exhibiting a normal distribution. Comparisons between groups were analyzed with the independent sample *t*-test. Enumeration data between groups was compared with the χ^2 test. Single-factor survival analysis and multivariate survival analysis were conducted by employing Kaplan–Meier, Log rank and Cox regression analyses. $P < 0.05$ was considered to be statistically significant.

Ethical Approval/Patient Consent

The present study was approved by the Ethics Committee of first affiliated hospital of Soochow University (number: 129/2020, dated 19/04/2020). All patients, control subjects, and their family members provided informed consent. All materials and methods conformed to correlative policies and regulations. This investigation was performed in accordance with the declaration of Helsinki.

Results

Clinical Data

The features of research population are shown in Table 1. Three groups showed greatly higher extents of ALT and AST than control group ($P < 0.01$). Other characteristics including age, gender, BMI or HCV-RNA content displayed no great differences.

Table 1 The Characteristic of Study Population

Variable	Control	CHC (N=48)	Liver Cirrhosis (N=43)	Hepatocellular Carcinoma (N=25)	P
Gender	39/31	28/20	22/21	12/13	0.824
Age	48.73±8.95	47.48±9.52	46.80±9.63	48.12±9.47	0.426
BMI	23.45±2.36	23.27±2.28	23.36±2.30	23.18±2.14	0.580
Family history [n(%)]	4(5.7)	3(6.3)	2(4.7)	2(8.0)	0.536
History of blood transfusion [n(%)]	6(8.6)	5(10.4)	5(11.6)	4 (16.0)	0.374
Surgical history [n(%)]	3(4.3)	3(6.3)	4(9.3)	5(20.0)	0.185
History of blood donation [n(%)]	10(14.3)	4(8.3)	4(9.3)	3(12.0)	0.408
History of alcohol consumption [n(%)]	25(35.7)	18(37.5)	20(46.5)	12(48.0)	0.103
Smoking history [n(%)]	20(28.6)	15(31.3)	14(32.6)	8(32.0)	0.442
ALT (U/L)	17.13±5.81	94.65±26.72 ^a	101.15±30.64 ^a	97.28±29.42 ^a	<0.01
AST (U/L)	25.16±7.42	88.70±20.95 ^a	93.86±25.10 ^a	91.65±22.37 ^a	<0.01
HCV-RNA (IgIU/mL)	Not detected	6.53±1.70	6.24±1.58	6.60±1.51	0.203

Note: Compared with control, ^a $P < 0.01$.

Abbreviations: CHC, chronic hepatitis C; HCC, hepatocellular carcinoma.

Table 2 miR-155 and miR-224 Expression Level in Different Groups

Variable	Control (N=70)	CHC (N=48)	Liver Cirrhosis (N=43)	HCC (N=25)
miR-155	0.24±0.06	0.97±0.35 ^a	2.05±0.41 ^{ab}	4.08±0.92 ^{abc}
miR-224	0.12±0.03	0.81±0.28 ^a	1.86±0.31 ^{ab}	3.87±0.75 ^{abc}
AFP (ng/mL)	9.73±2.80	45.78±10.32 ^a	51.70±11.41 ^a	364.26±35.65 ^{abc}

Notes: Compared with control, ^aP<0.05; Compared with CHC, ^bP<0.05; Compared with liver cirrhosis, ^cP<0.05.

Abbreviations: HCC, hepatocellular carcinoma; CHC, chronic hepatitis C.

There was no significance in parameters including age, gender, BMI or HCV-RNA content. Compared with control group, significantly higher extents of ALT and AST were detected in CHC, liver cirrhosis and hepatocellular carcinoma.

Expression Levels of miR-155 and miR-224

The expression extents of serum miR-155 and miR-224 increased along with the increase of malignancy (all P<0.05). The detailed information is shown in Table 2.

For both miR-155 and miR-224, gene expression is highest in HCC patients, followed by cirrhosis and HCV patients, and lowest in healthy people.

The Values of miR-155 and miR-224 for HCC Diagnosis

The area under the curve of miR-155 and miR-224 in the combined diagnosis of HCC was higher than that of single miR-155 and miR-224, the diversity was of statistical significance ($z=4.107$, 4.315 , $P=0.016$, 0.005), the sensitivity was 93.0%, and the specificity was 86.2%. There was no significant difference in the region under the curve of miR-155 in the diagnosis of HCC compared with miR-224 ($z=1.108$, $P=0.217$). The detailed information was shown in Table 3 and Figure 1.

Relationship of Expression Level Between miR-155 and miR-224

According to relationship analysis, the expression level of miR-155 was positively related to miR-224 in CHC group ($r=0.716$, $P<0.001$). The similar phenomenon was also occurred in miR-155 and miR-224 in HCC group ($r=0.784$, $P<0.001$). The detailed information was shown in Figures 2–4.

Discussion

CHC is the most important cause of end-stage liver disease in Europe, the United States and Japan, according to the World Health Organization statistics, there are more than 170 million patients infected with HCV worldwide.^{34,35} Cirrhosis due to chronic HCV infection is the primary index for liver transplantation and a major factor in the increased incidence of HCC.^{7,8,36–38}

MiRNA is a cluster of about 21–25 nucleotides, which regulates gene expression through targeting mRNA. miRNA is small, conserved, noncoding and endogenous. miRNA is involved in a variety of biological and pathological processes such as cell development, signal transduction, cell growth, proliferation, movement and apoptosis.^{7,8,34} It is common that miRNAs is expressed abnormally. miRNA is a kind of endogenous non-coding single stranded small molecules with a length of about found in eukaryotes in recent years. It can combine with the corresponding target genes through base

Table 3 Serum miR-155 and miR-224 Cutoff Points and the Corresponding Sensitivity and Specificity for Predicting HCC

Indicator	Cutoff Point	AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR	-LR
miR-155	1.57	0.847(0.785~0.908)	86.0	79.6	82.4	83.5	3.888	0.209
miR-224	1.25	0.813(0.755~0.872)	83.5	75.2	79.0	80.4	4.398	0.172
Combinations	/	0.918(0.856~0.974)	93.0	86.2	88.4	91.3	7.008	0.086

Abbreviation: HCC, hepatocellular carcinoma.

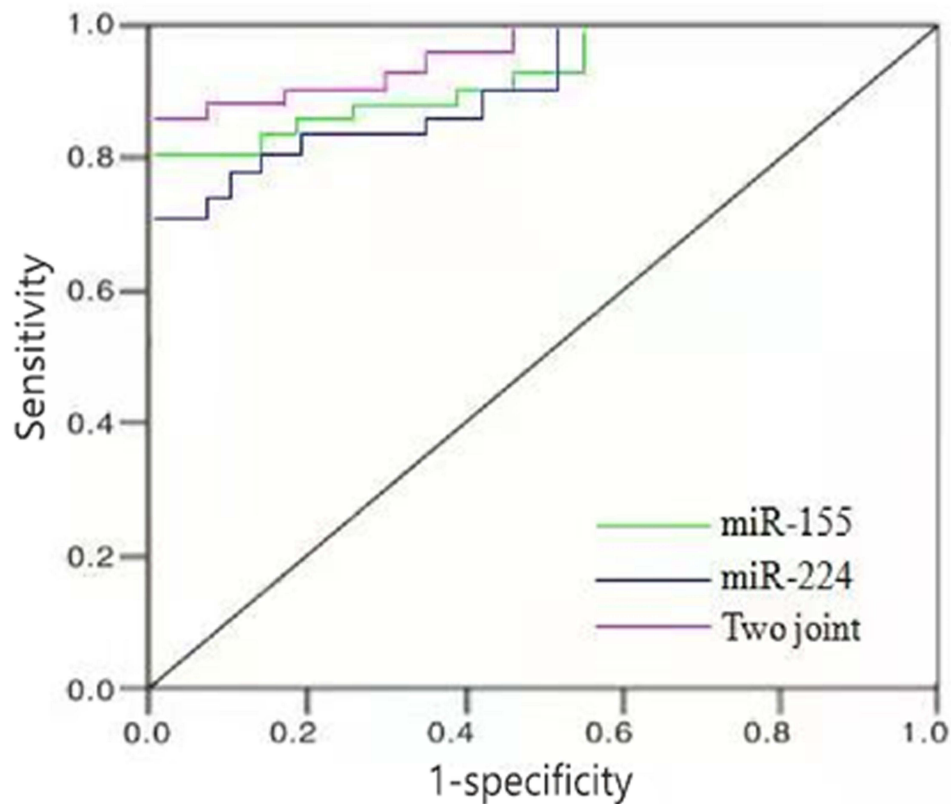


Figure 1 ROC curve of miR-155 and miR-224 combined in the diagnosis of HCC.

Abbreviation: HCC, hepatocellular carcinoma.

pairing, resulting in target degradation or post-transcriptional translation inhibition, and control gene expression at the post-transcriptional extent. Studies have shown that it not only takes part in a lot of biological processes including cell division, proliferation, differentiation and development but also plays a function similar to oncogenes and tumor suppressor genes, and exerts a significant effect on tumor cell proliferation, apoptosis, differentiation and metabolism.^{39–44} MiRNAs regulate and control the formation and development of tumors by interacting with various signaling pathways such as EGFR/STAT3, JAK2/STAT3, NF- κ B, AKT/mTOR. Recently, several possible key player targets in cancer that have been linked to various cancer types such as Akt, Topo-isomerase II-B, CTLA4, STAT4, FXR.^{45–48}

MiRNA exerts a significant effect on the development of CHC, hepatocyte regeneration and functional regulation as a hidden marker for diagnosing and treating HCC. miR-155 and miR-224 have been extensively studied in various types of diseases and multiple countries and regions, especially in China. It has been found that aberrant expression of miR-155 displays as an oncogenic feature in various types of hematological malignancies and solid tumors.^{49–52} Furthermore, it has been studied in multiple inflammatory diseases including asthma,⁵³ sepsis,⁵⁴ periodontitis⁵⁵ and rheumatoid arthritis.⁵⁶ miR-224 regulates the expression of upstream and downstream genes and affects the occurrence and progression of diseases including liver cancer,⁵⁷ colorectal cancer,⁵⁸ uveal melanoma⁵⁹ and cervical cancer.⁶⁰ Zhang et al have shown that miR-155 participates in developing and differentiating immune cells with high expression in HCC tissues.⁶¹ It can promote the appearance, invasion and metastasis of HCC cells, and exerts a significant effect on the prognosis and targeted treatment of HCC. Cui et al

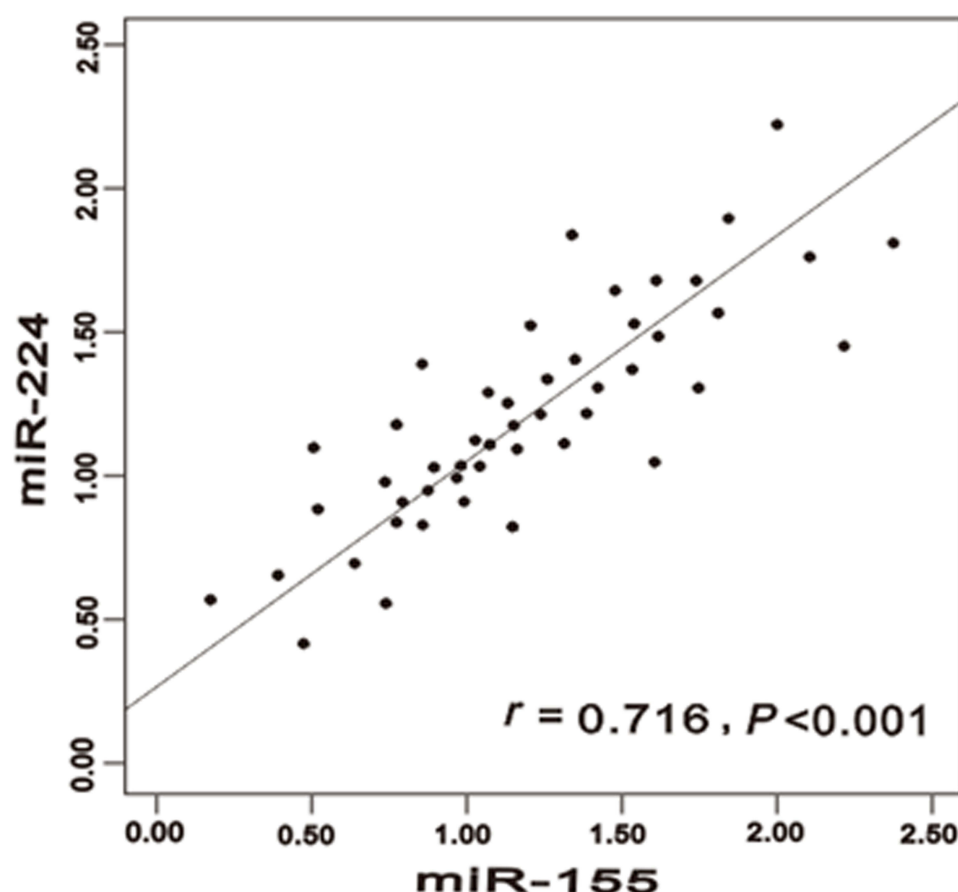


Figure 2 Correlation between miR-155 expression level and miR-224 in CHC.

Abbreviation: CHC, chronic hepatitis C.

found that miR-224 is the promoter of HCC, and its expression in hepatocellular carcinoma is related to tumor cell propagation, migration, aggression and prognosis, which can be used as a diagnostic and prognostic index of HCC.⁶²

According to this study, by comparing with the control group, miR-155 and miR-224 were remarkably increased in HCC group, liver cirrhosis group and CHC group, and the HCC group was noticeably higher than CHC group and liver cirrhosis group. To the best of our knowledge, the relationship between miR-155 and CHC infection has been reported in only a few literatures in Caucasian population. And the relationship between miR-224 and CHC infection has not been reported. It is suggested that the high expression of miR-155 and miR-224 may take part in occurring and developing hepatitis C. Some studies have found that the level of serum miR-224 in patients suffering from HCV-related hepatitis and HCC is related to the grade of hepatitis, the degree of liver fibrosis and the degree of tumor tissue differentiation.^{63,64} Monitoring the expression of miR-224 may be helpful to diagnosing and treating response evaluation of patients suffering from HCC. The ROC curve of this study indicates that the combination of miR-155 and miR-224 has the largest area under the curve for the diagnosis of HCC, with sensitivity of 93.0% and specificity of 86.2%. Correlation analysis also showed that miR-155 and miR-224 in HCC group are significantly positively correlated. This demonstrates that miR-155 and miR-224 may be biological indicators for diagnosing HCC, and the integrated detection of miR-155 and miR-224 has good values in the diagnosis of HCC. Researches have shown that the expression extent of miR-155 in HCC tissues and serum

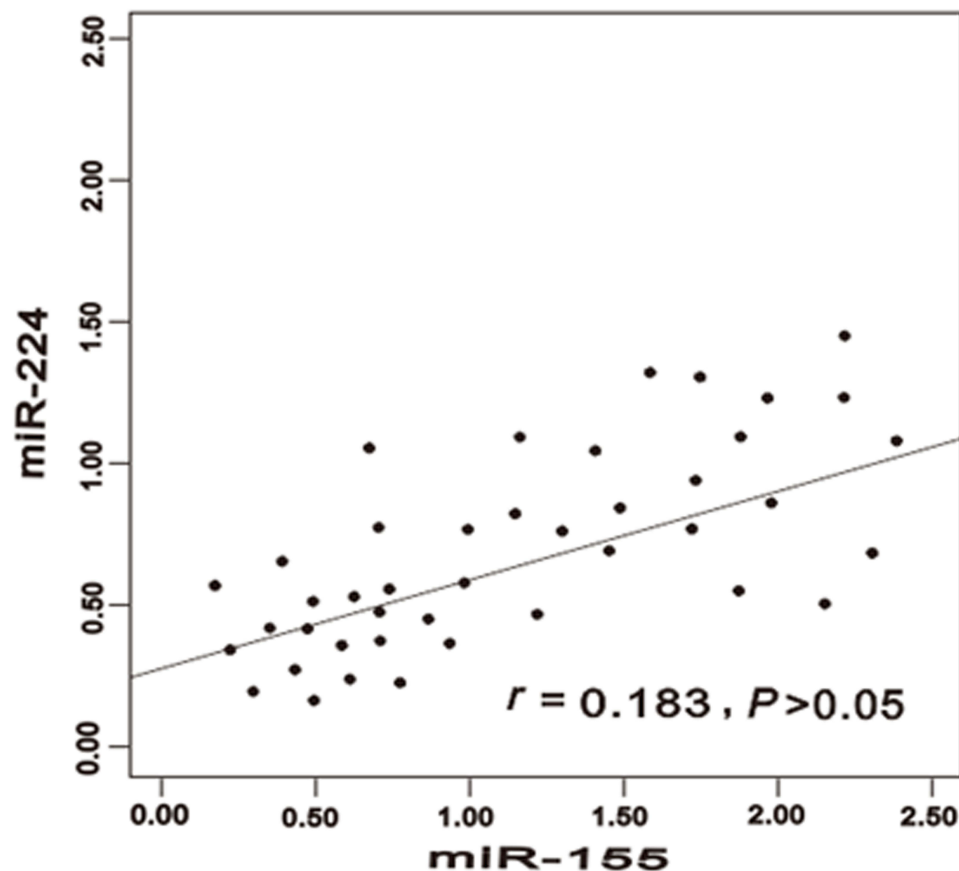


Figure 3 Correlation between miR-155 expression level and miR-224 in liver cirrhosis.

is greater than that in normal liver tissues and healthy controls.⁴² The region under the curve of miR-155 in diagnosing HCC is 0.840, which has high efficiency in the diagnosis of HCC and may be a good index for the diagnosis of HCC. Amr et al observed that the extent of miR-224 in HCC group was greatly higher than that in the CHC group and control group, which was associated with the appearance and growth of HCC.⁶³ The expression of miR-224 could be adopted as a noninvasive biomarker for early prediction of HCC. According to other researches, the level of serum miR-224 in patients with early HCC is significantly higher than that in patients suffering from liver cirrhosis, chronic hepatitis and healthy controls.^{41,42} The area under the curve for identifying early HCC is 0.880. miR-224 is a reliable biomarker for the early detection of HCC. It should be noted that some useful molecular techniques used such as microarrays and RNA-Seq should be applied to detect miRNAs in the future.^{65–69}

Conclusion

In summary, with the progress of the disease, the expression levels of serum miR-155 and miR-224 among patients suffering from hepatitis related to HCV infection increased strikingly. miR-155 and miR-224 are remarkably increased in patients suffering from HCV infection. The combination of miR-155 and miR-224 has a good diagnostic value for HCC caused by HCV infection.

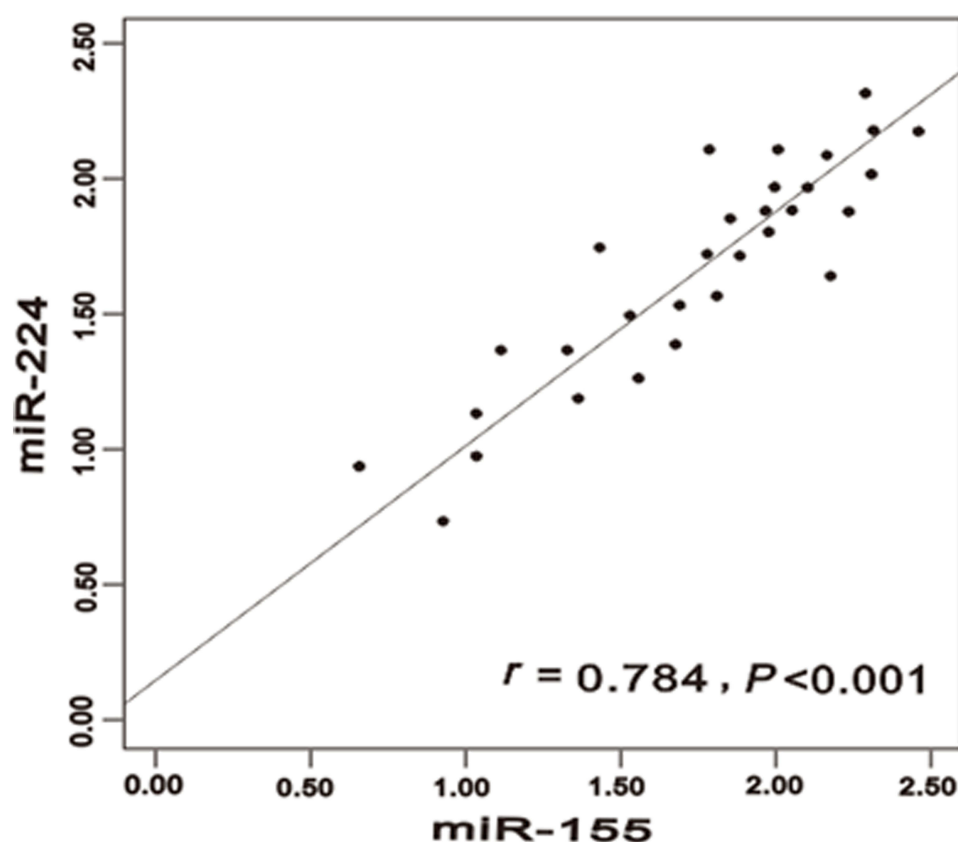


Figure 4 Correlation between miR-155 expression level and miR-224 in HCC.

Abbreviation: HCC, hepatocellular carcinoma.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Ethical Statement

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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Author Contributions

YZ conceived study design; XJ and YZ conceived the content concept; HW, XJ and YZ performed the data collection, extraction and analyzed the data. HW, XJ and YZ interpreted and reviewed the data and drafts. YZ and HW reviewed the final draft. All authors were involved in literature search, writing the paper and had final approval of the submitted and published versions. All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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