The preliminary data of gene expressions and bioinformatics analysis of miR-146b-5p and miR-4510 in Turkish population in HBV related hepatocellular carcinoma

RUNNING HEAD: *In-vivo* and *in-silico* miRNA analysis in HCC

Duygu Bircan Kadioglu[1,2], 0000-0002-5956-9774

Coskun Ozer Demirtas[3], 0000-0002-0004-2740

Dilek Pirim[4], 0000-0002-0522-9432

Feyza Dilber[3], 0000-0003-2901-7044

Fatih Eren[1,5,6], 0000-0001-8126-2413

[1]Department of Medical Biology, School of Medicine, Marmara University, Istanbul, Turkiye

[2] Institute of Health Sciences, Marmara University, Istanbul, Turkiye

[3]Department of Gastroenterology, School of Medicine, Marmara University, Istanbul, Turkiye

[4]Department of Molecular Biology & Genetics, Faculty of Arts and Sciences, Bursa Uludag University, Bursa, Turkiye

[5]Department of Medical Biology, School of Medicine, Recep Tayyip Erdoğan University , Rize, Turkiye

[6] Institute of Gastroenterology, Marmara University, Istanbul, Turkiye

Corresponding author: Fatih EREN, Department of Medical Biology, School of Medicine, Marmara University, Basıbuyuk, Basıbuyuk Yolu No: 9 D:2, 34854 Maltepe/Istanbul, Turkey, E-mail: fatiheren@marmara.edu.tr

Abstract

Objective: It is reported that miRNAs play an important role in hepatocellular carcinogenesis and may serve as a non-invasive biomarker for hepatocellular carcinoma (HCC). MiR-4510 and miR-146b-5p expression levels were found to be associated with HCC, however their associations with hepatitis B virus (HBV) related HCC (HBV-HCC) is yet to be explored. We aimed to assess the predictive value of expression levels of serum miR-4510 and miR-146b-5p in patients with HBV-HCC and performed bioinformatics analyses based on the miRNA expression profile.

Methods: This cross-sectional study used serum of 16 patients with Chronic Hepatitis B (CHB), 15 hepatitis B virus related cirrhosis (HBV-cirrhosis), 15 HBV-HCC and 16 healthy subjects. The total RNA was isolated from serum and the expression of miRNAs were measured by qRT-PCR calculated using $2^{-\Delta\Delta Ct}$ methods. MIENTURNET was used to predict miRNA-target genes interactions. The Network Analyst was used to build protein-protein interaction.

Results: There was a significant difference in miR-146b-5p between study groups (P=0.009). MiR-146b-5p expression was found to be significantly reduced in HBV-HCC

compared to HBV-cirrhosis group and healthy controls. (*P*=0.005 and *P*=0.006, respectively).

Conclusion: The serum miR-146b-5p levels might be a promising tool to be used as a non-invasive diagnostic biomarker for HCC. Our findings shed light on potential biomarkers for the diagnosis of HBV-HCC in terms of selected miRNAs. The target pathways of miR-146b-5p identified by our *in-silico* analysis to reveal the functional mechanism are “MAPK signaling pathways" and "Pathways in cancer".

Keywords: Hepatocellular Carcinoma, HBV, miRNA, Biomarker, Cancer

Introduction

Hepatocellular Carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer mortality [1]. HCC is defined as a primary tumor in the liver, mainly in patients suffering from chronic liver cirrhosis or hepatitis B or C [2]. According to the World Health Organization, Turkey has intermediate (2–8%) endemicity for hepatitis B virus (HBV) [3]. After several decades of chronic HBV infection, approximately 20-30% of cases with cirrhosis develop HCC [4]. Also, there is a group of HBV-infected cases without cirrhosis who develop HCC [5]. In both cases, high tumor burden is associated with a poor prognosis. Thus, early detection of tumors at a curative stage increases the chances of successful treatment and long-term survival [4]. However, the development of non-invasive diagnostic tools for early detection of HCC is still a major challenge [6].

To diagnose and to determine the prognosis of HCC has been settling down as a paradigm in clinical practice. Serum alpha fetoprotein (AFP) is the most widely used tumor marker in detecting patients with HCC, and has been proven to have capability of prefiguring the prognosis [7]. Numerous non-invasive biomarkers have been investigated for their potential role to increase or surpass the diagnostic and prognostic utility of AFP for HCC. Recently, miRNAs which regulate gene expression both the transcriptional and translational

levels have been reported to be potential biomarkers for HCC diagnosis, [8]. Existing literature shows that miRNAs are more stable than mRNA and clinical application of miRNA detection can be easily used in non-tissue specimens like plasma or serum. Some miRNAs are differentially expressed in serum samples of HBV related HCC, CHB patients, and healthy individuals have been reported [9,10]. Additionally, accumulating evidence indicates that miRNAs can act as oncogenic, or tumor suppressive factors involved in HCC progress [11,12].

It is reported that down-regulation of miR-146b-5p in HCC tissues was related to malignant clinical features and poor prognosis. Using *in vitro* and *in vivo* studies, miR-146b-5p was demonstrated as a novel inhibitor for tumor growth and metastasis in HCC. The multiple anti-cancer functions of miR-146b-5p were due to the inhibition of the *TRAF6*/p-Akt pathway. Together, researchers suggested that miR-146b-5p could become a new prognostic biomarker and potential therapeutic target in HCC [13]. There is also a study about another miRNA, namely mir-4510, that it can be a novel biomarker for HCC. It has been demonstrated that miR-4510 may acts as a tumor suppressor in the liver by targeting many proto-oncogenes. *Glypican-3* (*GPC3)* is one of the numerous oncogenes overexpressed in HCC and clearly constitutes a relevant molecular target [14]. MiR-4510 is the most potent inhibitor of *GPC3* in HCC cells acted as tumor suppressors in liver by inhibiting tumoral cell growth and proliferation [14]. In the HCC, the other molecular targets of mir-4510 are *GPC3* and *RAF1*, and subsequently controlling key biological and signaling pathways among which *Wnt* and RAS/RAF/MEK/ERK signals [15]. Moreover, its tumor suppressor potential has been also shown in other cancer types. For instance, the downregulation of miR-4510 might promote the progression of gastrointestinal stromal tumors (GISTs) by increasing *APOC2*

expression [16]. Considering all these facts, the miR-146b-5p and miR-4510 has been proposed as two promising biomarkers to have a potential of utilization in the diagnosis of HCC.

The purpose of this study is to investigate the prediction value of miR-146b-5p and miR-4510 by detection their serum expression level in the HBV related-HCC (HBV-HCC), HBV related-Cirrhosis (HBV-Cirrhosis), CHB and healthy subjects. Moreover, integrative bioinformatic analyses were conducted to reveal the molecular mechanisms of selected miRNAs by identifying target gene and their protein-protein interaction (PPI) networks. Our study presents both *in silico* and *in vivo* findings regarding miRNA/target genes for the prediction of HBV-related HCC.

Materials and Methods

Patients and Serum Samples

Serums were collected from the patients aged 18 years and older with HBV-HCC (n=15), HBV-cirrhosis (n=15), CHB (n=16), and liver-disease free healthy controls (n=16) from XXX University, Institute of XXX,. The patients' clinical, laboratory and tumoral characteristics were extracted from the prospectively collected database for XXX University. The control group members had no evidence of hepatocellular or other cancers, history of liver disease, serological evidence of hepatitis B or C infection, nor any kind relationship to the experimental group. Each control was pair-matched by sex and age (±3 years). All participants were of Turkish ancestry. The participant's recruitment was approved by the Committee for Ethics of Medical Experiments on Human Subjects, the Faculty of Medicine in XXX University (Approval no:09.2020.1153, Date:06.11.2020). All patients and controls

provided informed consent to participate in the study. The study was performed in accordance with the principles of the Helsinki Declaration.

The serum samples collected from biobank were centrifuged at 12,000 rpm for 15 min at 4°C, then, the serum supernatants were carefully transferred into RNase-free tubes and stored at -80°C for further analyses. The serum samples of patients with HBV-HCC, HBV-cirrhosis, and CHB examined for miR-146b-5p and miR-4510 expression profiles to provide potentially important non-invasive biomarkers to be used for early diagnosis of HCC.

Total RNA Extraction from Serum

Total RNAs were isolated using miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) from serum samples according to the manufacturer's protocol. The concentration and purity of RNA were determined spectrophotometrically based on the absorption at 260 to 320 nm. We assessed the RNA concentration using a Biotec Synergy H1 spectrophotometer and RNA was stored at -80 °C for expression analysis.

cDNA synthesis

cDNA synthesis was performed using the miRCURY® LNA® RT kit according to the manufacturer's instructions, using Applied Biosystem BioRad C1000 Touch Thermal Cycler device. Cycler conditions were as follows: reverse-transcription 60 mins, 42ºC and inactivation 5 mins, 95ºC.

Quantitative real-time PCR (qRT-PCR)

The levels of miR-4510 and miR-146b-5p in serum were measured using the miRCURY LNA SYBR® Green PCR Kit by real-time PCR using a LightCycler480 II Sequence Detection System (Roche, Basel, Switzerland). All assays were performed in 96-well plates, including negative template controls. Relative miR-4510 and miR-146b-5p

expression were calculated using the comparative cycle threshold (Ct) method. All RT-PCR experiments were run in duplicate and *SNORA66* was used as housekeeping gene for miRNAs expression analysis. All steps followed the manufacturer's suggested protocol. $2^{-\Delta\Delta Ct}$ method was used to quantify miRNAs in serum.

Data collection and prediction target genes of miR-146b-5p

Target genes of miR-146b-5p were retrieved from miRTargetLink Human databases (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2) which give miRNA-gene interactions using miRCarta, miRPathDB, miRbase, miRNA Tissue Atlas, miRTarbase databases [17].

Target gene pathway enrichment and network-based analysis of miRNAs

The MIENTURNET (http://userver.bio.uniroma1.it/apps/mienturnet/) web tool was used to assess the miRNA-target interactions and perform network-based analyses [18]. The Network Analyst (https://www.networkanalyst.ca/) is a comprehensive gene-centric platform supporting gene expression profiling, biological network analysis and visual exploration. Gene set overrepresentation analysis (ORA) is one method of exploring the biological meaning of computationally defined modules. Imex interactom database was selected to contracted of PPI network via generic PPI in Network analyst platform. In the function enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) terms with $P<0.05$ according to the hypergeometric test were considered statistically significant. Each significantly enriched gene set from Gene Set Enrichment Analysis (GSEA) is represented as a node. Gene sets with overlapping genes are connected with an edge (calculated using the overlap coefficient or Jaccard index). The network visualization simplifies the interpretation of GSEA results by grouping similar gene sets together. This

platform was used to perform genetic protein-protein interaction pathway and global enrichment analysis of miR-146b-5p human target genes obtained from miRTargetLink Human as described earlier [19]. The genes with the higher betweenness centrality and degree were determined as the most important genes in these network (hub genes). Eventually, the function of the hub genes was identified by KEGG mapper.

In the present study, we performed the RT-PCR to evaluate miRNA expression levels and a bioinformatics analysis on the miRNA/target gene enrichment on HCC. We also collected LIHC data from TCGA and these in silico samples were used to determine which genes targeted miR-146b-5p in HCC. Finally, a potential relationship between miR-146b-5p levels and targeted genes' KEGG pathway was investigated. KEGG pathway analysis were used to gain further insights into the pathways significantly involved by miRNAs. KEGG pathway analysis in the present study demonstrated that differentially expressed miRNAs were mainly enriched in the pathways in cancer. Based on the experimental certificate miRNA-target gene databases before mentioned, the miRNA-target gene regulatory network was constructed using Network Analyst web tool.

Statistical Analysis

The median and interquartile range (IQR) were used to display continuous skewed data, and mean standard deviation (SD) was used if normally distributed. The results of categorical data are given as absolute numbers with percentage. For the comparison of continuous variables between four groups, Kruskal-Wallis test was used when the data conformed to an abnormal distribution; and Mann-Whitney U test was used for the post-hoc analysis between two groups. Chi-square test was used to compare categorical parameters. Logistic regression analysis was used to investigate potential association of miR-

146b-5p with CHB presence and progression. Spearmen's rho test was performed to investigate a possible correlation between miR-146b-5p. To reveal the association of miR-146b-5p with survival in HCC patients, we performed univariate Cox-regression analyses. The statistical significance was defined as $P<0.05$. All statistical analyses were conducted using the SPSS software version 20.0 (IBM, Armonk, NY, USA).

Results

Determining the Characteristics of the Groups

A total of 46 HBV patients and 16 healthy subjects were recruited into the present study. Baseline characteristics and clinicopathological parameters are presented in Table 1. Overall, HBV patients were older than healthy controls ($P=0.001$). The gender distribution was indifferent among all groups as well as body-mass index (BMI) levels, presence of diabetes and smoking. The HBV-HCC patients had higher median Child-Pugh score comparing to the HBV-cirrhosis (7 vs 5, $P=0.031$), while the median Model for End-Stage Liver Disease (MELD) scores were not statistically different. Complications of cirrhosis, including ascites, esophageal varices, history of variceal bleeding and hepatic encephalopathy was not different among HBV-cirrhosis and HBV-HCC groups. As expected, the median alpha-fetoprotein (AFP) levels were higher in HBV-HCC group.

Relative quantification of miR-146b-5p in each group

The goal of the present study was to explore the potential use of serum miR-146b-5p and miR-4510 as biomarkers for HCC. In this marker discovery phase, all reactions were studied in duplicate and ΔCt values were calculated for miR-146b-5p using housekeeping gene the *SNORA66*. The quantitively expression values ($2^{-\Delta\Delta Ct}$ values) of all groups are shown in Figure 1. It was observed that ΔCt values in the groups were not in accordance with

the normal distribution. For this reason, the difference was examined with the Kruskal Wallis test. Expression levels of miR-146b-5p in serum were found significantly lower in the HBV-HCC group when comparing to HBV-cirrhosis, CHB and healthy control groups. The expression levels of miR-146b-5p and their comparison among groups are exhibited in Table 2. A significant difference was found between the groups regarding the miR-146b-5p levels (P=0.009) (Table 2). Also, significant differences were found when the HBV-HCC group was compared with the HBV-Cirrhosis (P=0.006) and the healthy control group (P=0.005), respectively (Table 3).

We further examined the correlation between the expression of serum miR-146b-5p with clinical parameters. No significant association was found between the miR-146b-5p and clinicopathological parameters (all P values >0.05).

As for miR-4510, the expression was seen only in three individuals in the control group by RT-qPCR, thus, it was not included in further analytic studies. miR-4510 was not expressed in HBV-HCC, HBV-Cirrhosis and CHB groups. Both statistical analysis and bioinformatic analysis were not carried out due to the lack of a quantitative value of miR-4510 that could be compared between the groups.

Prediction of target genes of miR-146b-5p

The target gene network was constructed as validated genes and "1" was accepted as a minimum shared target, using the miRTargetLink Human Database. Results showed 120 genes were targets of miR-146b-5p, and Table 4 representing these validated target genes.

Pathway enrichment and network-based analysis of miR-146b-5p

Network Analyst was used to perform pathway enrichment analysis of target genes of miR-146b-5p obtained from miRTargetLink Human database as described earlier. The

one hundred twenty target genes of miR-146b-5p were run on the Network analyst platform to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment. The results were displayed according to their *P*-values. Sixty-two pathways had $P<0.05$ value for KEGG pathway enrichment analysis of miR-146b-5p; "MAPK signaling pathway" and "Pathway in cancer" were the most statistically significant pathway (Figure 2). Ten significant HCC related pathways were listed in Table 5.

Discussion

It is well known that miRNAs play a number of roles in the various cancer types including HCC [20,21]. However, the potential of miRNAs as biomarkers for the detection of HCC is still unexposed. In the literature, it has shown the anti-cancer functions of miR-146b-5p and miR-4510 on HCC and other cancer types. Therefore, it can be speculated that these miRNAs may be a novel promising biomarker for prediction of HCC [22]. Moreover, there are no sufficient in-vivo studies investigating the association between these miRNAs and HCC related HBV, although chronic HBV patients are more than 100 times more likely to develop HCC than those who are not infected [23]. Our aim was to estimate the effect of expression levels among various sub-groups (CHB, HBV-Cirrhosis and HBV-HCC) to determine the role of these miRNAs in HBV-HCC. In our study, the expression of mir-146b-5p was found to be relatively decreased in the serum of patients with HBV-HCC, for the first time. Moreover, it seems that miR-146b-5p expression was not related with HBV infection. Our other interesting result is that miR-4510 cannot be detected in the serum in comparable

levels in all study groups. These results suggest that miR-146b-5p can be helpful in the diagnosis of HCC, but not in pre-cancerous HBV infection. Our finding that miR-146b-5p is associated with HCC independently of HBV should be questioned in further studies. Our study is not sufficient to reveal the mechanism underlying this finding.

It is anticipated that circulating miRNAs are also affected during HCC progression because many miRNAs are dysregulated in the occurrence process of HCC. Therefore, more research across a range of populations and stages of HCC have been accomplished [24]. miR-146b-5p has the most marker genes regulating HCC, and may be a promising new biomarker for predicting HCC prognosis [22].

To identify a predictive biomarker, we analyzed the expression levels of miR-146b-5p and miR-4510 in HBV-HCC. We studied these levels in different sub-groups (CHB, HBV-Cirrhosis, and HBV-HCC) to determine the role of these miRNAs in HBV progression. Our study confirms past research on cancer that showed lower expression of miR-146b-5p in HBV-HCC patients compared to other groups, and higher expression in the control group [13]. In our study, the expression of mir-146b-5p was found to be relatively decreased in the serum of patients with HBV-HCC which makes it more practical for use in clinical practice. Similarly, several other miRNAs were found to be down-regulated in the serum of HCC patients [25]. The study discovered that miR-146a, which is present in HCC, may help to suppress the growth and spread of HCC cells. Overexpression of miR-146a can also inhibit HCC cell proliferation and invasion.[26]. Based on this data, we hypothesize that miR-146b-5p acts as a tumor suppressor. In addition to its expression in tumor tissues, serum concentrations of miR-146b have also been studied in patients with other types of cancer [27]. Studies have found that when miR-146b-5p is reduced in HCC, it can negatively impact cell

growth and metastasis [13]. However, miR-146b-5p was reported to be a tumor suppressor or oncomiRNA in various types of cancer and some studies suggested that increasing miR-146b-5p expression enhances cell proliferation, metastasis, invasion, and worse overall survival suggesting that miR-146b serves as a tumor suppressor [28]. The results were contradictory in other tumor types. Thus, miR-146b-5p may play oncogenic or tumor suppressive roles depending on the tissue type and specific targets.

Note that miR-4510 may not be found in some people's serum after HBV infection. In cancer patients, miRNAs can be found in body fluids at different levels than in healthy people [29]. Our study found that miR-4510 was only found in the control group, possibly because it inhibits the expression of *glypicane-3* (*GPC3*), which is decreased in HCC tumors [14]. MiR-4510 is the most powerful inhibitor of *GPC3* expression, which can stop the growth and cause cell death in HCC cells. It can also reduce the growth, migration, and invasion of GIST-882 cells [16]. It inhibits HCC cell growth, movement, and causes senescence by reducing *RAF1* expression. MiR-4510 regulates proto-oncogenes and important signaling pathways in HCC cells, including Wnt and RAS/RAF/MEK/ERK signals [15].

In the *in-silico* part of our study, we also investigated the target genes of miR-146b-5p that may have predictive value. It was observed that miR-146b-5p was increased in TGCA-LIHC tumor tissue in comparison to normal liver tissues in miR-TV database version 18.0 ($P<0.05$), but the expression of miR-146b-5p in the serum was decreased in the *in-vitro* part of our study, which shows that it may be at different levels in tissue and serum in HBV-HCC. Also, miR-4510 expression levels were not significant between in TCGA-LIHC tumor tissue and normal tissues ($P>0.05$). (http://mirtv.ibms.sinica.edu.tw/index.php) (Data is not shown.)

miR-146b-5p targets and transcriptionally regulates genes involved in cell growth, oncogenesis, tumor suppression, and apoptosis, including *NFKB1, TRAF6, IRAK1*, and others. By downregulating *TRAF6*, miR-146a may help inhibit the growth and spread of HCC cells, making it a potential therapeutic target for HCC. High levels of *TRAF6* are linked to various signaling pathways involved in cancer, and affect tumor cell proliferation, survival, apoptosis, and invasion [30]. Our study suggests that miR-146b-5p is important for HCC development, and overexpression of *TRAF6* is associated with tumorigenesis. More research is needed to fully understand the role of miR-146b-5p and its target *TRAF6* in HCC, and to validate their predictions.

In this study, we used KEGG enrichment analysis to show that the target genes were regulated by miR-146b-5p. This miRNA is mainly involved in "MAPK signaling pathway" and "pathways in cancer", which are both important for the development of HCC. Our findings are supported by previous research that has shown that MAPK signaling promotes cell invasion and tumor progression [31]. The main limitation of our study is the small number of cases and specific to the Turkish population so our results cannot be generalized. However, our analysis of differentially expressed miRNAs in HCC samples showed the importance of miR-146b-5p and miR-4510 in gene regulation, which should be further studied to understand the development of HCC. The miRNA that regulates these genes may be crucial for HCC.

MiR-146b-5p and KEGG-identified cancer genes may be associated with HCC development. Considering that miR-146b-5p may acts as a tumor suppressor, it seems reasonable to expect its expression to be high in HCC to suppress cancer, but its expression was

found to be low. However, considering the loss of function of tumor suppressors in the general molecular mechanism of cancer, low expression is also a significant finding. However, uncovering the mechanism underlying low miR-146b-5p expression in HCC is a new research topic. However, miR-146b-5p's function in HCC is still unclear, and small sample sizes may explain inconsistent results. More research is needed to explore this concept and evaluate miR-146b-5p's potential as a prognostic biomarker for HCC patients. It's possible that miR-146b-5p expression is differentially regulated in HBV-related HCC, but other HCC etiologies require further study.

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References

1. McGlynn KA, London WT. The global epidemiology of hepatocellular carcinoma: present and future. Clin Liver Dis 2011; 15 (2):223-243, vii-x.

2. Bugianesi E. Non-alcoholic steatohepatitis and cancer. Clinics in liver disease 2007; 11 (1):191-207.

3. Gurol E, Saban C, Oral O, Cigdem A, Armagan A. Trends in Hepatitis B and Hepatitis C Virus among Blood Donors over 16 Years in Turkey. European Journal of Epidemiology 2006; 21 (4):299-305.

4. Song P-P, Xia J-F, Inagaki Y, Hasegawa K, Sakamoto Y, Kokudo N, et al. Controversies regarding and perspectives on clinical utility of biomarkers in hepatocellular carcinoma. World journal of gastroenterology 2016; 22 (1):262.

5. Velázquez RF, Rodríguez M, Navascués CA, Linares A, Pérez R, Sotorríos NG, et al. Prospective analysis of risk factors for hepatocellular carcinoma in patients with liver cirrhosis. Hepatology 2003; 37 (3):520-527.

6. Cao M-Q, You A-B, Zhu X-D, Zhang W, Zhang Y-Y, Zhang S-Z, et al. miR-182-5p promotes hepatocellular carcinoma progression by repressing FOXO3a. Journal of hematology & oncology 2018; 11 (1):1-12.

7. Zhou L, Liu J, Luo F. Serum tumor markers for detection of hepatocellular carcinoma. World J Gastroenterol 2006; 12 (8):1175-1181.

8. Parizadeh SM, Jafarzadeh-Esfehani R, Ghandehari M, Goldani F, Parizadeh SM, Hassanian SM, et al. MicroRNAs as potential diagnostic and prognostic biomarkers in hepatocellular carcinoma. Current drug targets 2019; 20 (11):1129-1140.

9. Xu J, Wu C, Che X, Wang L, Yu D, Zhang T, et al. Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. Mol Carcinog 2011; 50 (2):136-142.

10. Zhou J, Yu L, Gao X, Hu J, Wang J, Dai Z, et al. Plasma microRNA panel to diagnose hepatitis B virus–related hepatocellular carcinoma. Journal of clinical oncology 2011; 29 (36):4781-4788.

11. Liu Z, Wang Y, Dou C, Sun L, Li Q, Wang L, et al. MicroRNA-1468 promotes tumor progression by activating PPAR-γ-mediated AKT signaling in human hepatocellular carcinoma. Journal of Experimental & Clinical Cancer Research 2018; 37 (1):1-14.

12. Ye Y, Zhuang J, Wang G, He S, Zhang S, Wang G, et al. MicroRNA-495 suppresses cell proliferation and invasion of hepatocellular carcinoma by directly targeting insulin-like growth factor receptor-1. Experimental and therapeutic medicine 2018; 15 (1):1150-1158.

13. Li C, Miao R, Liu S, Wan Y, Zhang S, Deng Y, et al. Down-regulation of miR-146b-5p by long noncoding RNA MALAT1 in hepatocellular carcinoma promotes cancer growth and metastasis. Oncotarget 2017; 8 (17):28683.

14. Cartier F, Indersie E, Lesjean S, Charpentier J, Hooks KB, Ghousein A, et al. New tumor suppressor microRNAs target glypican-3 in human liver cancer. Oncotarget 2017; 8 (25):41211.

15. Ghousein A, Mosca N, Cartier F, Charpentier J, Dupuy JW, Raymond AA, et al. miR-4510 blocks hepatocellular carcinoma development through RAF1 targeting and RAS/RAF/MEK/ERK signalling inactivation. Liver International 2020; 40 (1):240-251.

16. Chen Y, Qin C, Cui X, Geng W, Xian G, Wang Z. miR-4510 acts as a tumor suppressor in gastrointestinal stromal tumor by targeting APOC2. Journal of Cellular Physiology 2020; 235 (7-8):5711-5721.

17. Kern F, Aparicio-Puerta E, Li Y, Fehlmann T, Kehl T, Wagner V, et al. miRTargetLink 2.0—interactive miRNA target gene and target pathway networks. Nucleic Acids Research 2021; 49 (W1):W409-W416.

18. Licursi V, Conte F, Fiscon G, Paci P. MIENTURNET: an interactive web tool for microRNA-target enrichment and network-based analysis. BMC bioinformatics 2019; 20 (1):1-10.

19. Xia J, Benner MJ, Hancock RE. NetworkAnalyst-integrative approaches for protein–protein interaction network analysis and visual exploration. Nucleic acids research 2014; 42 (W1):W167-W174.

20. Chi Y, Zhou D. MicroRNAs in colorectal carcinoma-from pathogenesis to therapy. Journal of Experimental & Clinical Cancer Research 2016; 35 (1):1-11.

21. Braconi C, Henry JC, Kogure T, Schmittgen T, Patel T. The role of microRNAs in human liver cancers. Semin Oncol 2011; 38 (6):752-763.

22. Ding W, Yang H, Gong S, Shi W, Xiao J, Gu J, et al. Candidate miRNAs and pathogenesis investigation for hepatocellular carcinoma based on bioinformatics analysis. Oncology Letters 2017; 13 (5):3409-3414.

23. Bosch FX, Ribes J, Cléries R, Díaz M. Epidemiology of hepatocellular carcinoma. Clin Liver Dis 2005; 9 (2):191-211, v.

24. Xu J, An P, Winkler CA, Yu Y. Dysregulated microRNAs in hepatitis B virus-related hepatocellular carcinoma: potential as biomarkers and therapeutic targets. Frontiers in oncology 2020; 10:1271.

25. Qi P, Cheng S-q, Wang H, Li N, Chen Y-f, Gao C-f. Serum microRNAs as biomarkers for hepatocellular carcinoma in Chinese patients with chronic hepatitis B virus infection. PloS one 2011; 6 (12):e28486.

26. Zu Y, Yang Y, Zhu J, Bo X, Hou S, Zhang B, et al. MiR-146a suppresses hepatocellular carcinoma by downregulating TRAF6. Am J Cancer Res 2016; 6 (11):2502-2513.

27. Paterson MR, Kriegel AJ. MiR-146a/b: a family with shared seeds and different roots. Physiol Genomics 2017; 49 (4):243-252.

28. Deng X, Wu B, Xiao K, Kang J, Xie J, Zhang X, et al. MiR-146b-5p promotes metastasis and induces epithelial-mesenchymal transition in thyroid cancer by targeting ZNRF3. Cellular physiology and biochemistry 2015; 35 (1):71-82.

29. Iacona JR, Lutz CS. miR-146a-5p: expression, regulation, and functions in cancer. Wiley Interdisciplinary Reviews: RNA 2019; 10 (4):e1533.

30. Li J, Liu N, Tang L, Yan B, Chen X, Zhang J, et al. The relationship between TRAF6 and tumors. Cancer Cell International 2020; 20 (1):1-12.

31. Daroqui MC, Vazquez P, Bal de Kier Joffé E, Bakin AV, Puricelli LI. TGF-β autocrine pathway and MAPK signaling promote cell invasiveness and in vivo mammary adenocarcinoma tumor progression. Oncology reports 2012; 28 (2):567-575.

Table 1. Baseline characteristics and clinicopathological parameters

	HBV-HCC (n=15)	HBV- Cirrhosis (n=15)	CHB (n=16)	Control (n=16)	*P* value
Age (Year), med (min-max)	56 (30-76)	54 (31-77)	59 (30-70)	44 (20-51)	**0.001***
Sex, n (%)					
Female	9 (60.0)	8 (53.3)	7 (43.8)	9 (56.3)	0.660
Male	6 (40.0)	7 (46.7)	9 (56.2)	7 (43.8)	
Body Mass Index, med (min-max)	26.7 (19.5-45.7)	30.1 (18.0-46.7)	30.1 (18.0-46.7)	-	0.587
Diabetes, n (%)	4 (26.7)	2 (13.3)	3 (18.8)	-	0.651
Smoking, n (%)	7 (46.7)	3 (20.0)	3 (18.8)	-	0.155
Child-Pugh score, med (min-max)	7 (5-9)	5 (5-10)	-	-	**0.031***
MELD score, med (min-max)	13 (7-19)	11 (7-14)	-	-	0.119
Ascites, n (%)	9 (64.3)	4 (26.7)	-	-	0.066
Esophageal varices, n (%)	7 (50.0)	8 (53.3)	-	-	0.858
Variceal bleeding history, n (%)	0 (0.0)	2 (13.3)	-	-	0.483
Hepatic Encephalopathy, n (%)	2 (14.3)	2 (13.3)	-	-	0.941
Alpha-fetoprotein (ng/mL)	8.9 (1.1-230140.0)	1.8 (0.8-4.3)	-	-	**0.003***

Table 2. Statistical association of expression of miR-146b-5p among groups

	HBV-HCC (n=15)	HBV-Cirrhosis (n=15)	CHB (n=16)	Control (n=16)	P-value
miR-146b-5p	7.568 (3.000-24.590)	17.876 (6.276-100.079)	16.055 (4.069-85.924)	18.317 (5.426-396.176)	**0.009***

* A significant difference was found between the groups regarding the miR-146b-5p levels.

HBV-HCC: HBV-related HCC, CHB: Chronic Hepatitis B, n:sample size

Table 3. Statistical analysis of miR-146b-5p in pairs

	Mean Rank Difference	*P*-value
HBV-HCC vs. HBV-Cirrhosis	-18.233	***P*=0.006**
HBV-HCC vs. CHB	-14.719	*P*=0.059
HBV-HCC vs. Control	-19.357	***P*=0.005**
HBV-Cirrhosis vs. CHB	3.515	*P*=0.476
HBV-Cirrhosis vs. Control	-1.124	*P*=0.793
CHB vs. Control	-4.638	*P*=0.454

HBV-HCC: HBV-related HCC, CHB: Chronic Hepatitis B

Table 4. Target genes for hsa-miR-146b-5p

miRNA	miR-146b-5p									
Total Target Genes	120									
Gene symbol	*ACTBL2*	*AGO1*	*AKAP8*	*AKT3*	*ALG10B*	*ARL8A*	*ATAD1*	*ATG9A*	*ATP13A3*	*AVL9*
	BCL7B	*BRWD1*	*BTN2A2*	*C16orf52*	*CARD11*	*CASR*	*CCDC6*	*CCDC83*	*CD300LB*	*CDC73*
	CENPU	*COPA*	*COX1*	*CYBRD1*	*CYTIP*	*DECR1*	*DGCR6L*	*EDEM3*	*EGFR*	*ELP2*
	ENTPD5	*ERBB4*	*ESD*	*FANCF*	*GPM6B*	*GPRIN2*	*GRAP2*	*GXYLT2*	*HAAO*	*HNRNPD*
	HORMAD2	*HSPA1B*	*IL1RAP*	*IL1RL2*	*IL6*	*IRAK1*	*KCTD15*	*KDM6B*	*KIT*	*LBR*
	LIMD2	*LSM4*	*MALAT1*	*MAN1C1*	*MBD4*	*MDN1*	*METTL7A*	*MFSD6*	*MICAL2*	*MKRN2*
	MMP16	*MPP2*	*MRPL10*	*MRPS30*	*MYLK*	*MYO6*	*NACC1*	*NFKB1*	*NOVA1*	*NSFL1C*
	NSL1	*OR8U1*	*PACS2*	*PARD6B*	*PAX8*	*PDGFRA*	*PLA2G4A*	*PLEKHG5*	*PMAIP1*	*POMT2*
	POU3F1	*PPP1R11*	*PPWD1*	*RAB2B*	*RARB*	*RGS9BP*	*RHOA*	*RHOBTB3*	*RUFY2*	*S100A12*
	SERBP1	*SERPINA4*	*SERTAD2*	*SFRP1*	*SHCBP1*	*SLC10A3*	*SLC5A5*	*SQSTM1*	*SRPRB*	*ST6GAL2*
	TLL1	*TLR4*	*TMEM101*	*TMEM136*	*TMEM167A*	*TMEM214*	*TMPRSS5*	*TRAF6*	*UHRF1*	*UMPS*

UHRF1 UMPS USP48 UTP15 WSB2 XPO4 ZNF117 ZNF260 ZNF292 ZNRF3

Table 5. Ten most significant HCC-related KEGG pathways for miR-146b-5p

Name	Hits	*P*-value	Adjusted *P*-value (FDR)	Genes in pathway
MAPK signaling pathway	11/295	2.51e-6	7.97e-4	*EGFR, ERBB4, NFKB1, TRAF6, IRAK1, HSPA1B, PLA2G4A, AKT3, KIT, PDGFRA, IL1RAP*
Pathways in cancer	13/530	2.68e-5	0.00236	*EGFR, NFKB1, TRAF6, AKT3, KIT, PDGFRA, IL6, RHOA, PAX8, RARB, PLEKHG5, PMAIP1, CCDC6*
Toll-like receptor signaling pathway	6/104	5.63e-5	0.00298	*NFKB1, TRAF6, IRAK1, AKT3, IL6, TLR4*
Phospholipase D signaling pathway	6/148	3.92e-4	0.0114	*EGFR, PLA2G4A, AKT3, KIT, PDGFRA, RHOA*
NF-kappa B signaling pathway	5/100	4.78e-4	0.0114	*NFKB1, TRAF6, IRAK1, TLR4, CARD11*
HIF-1 signaling pathway	5/100	4.78e-4	0.0114	*EGFR, NFKB1, AKT3, IL6, TLR4*
T cell receptor signaling pathway	5/101	5.01e-4	0.0114	*NFKB1, AKT3, RHOA, CARD11, GRAP2*
Hepatitis B	6/163	6.55e-4	0.0139	*NFKB1, TRAF6, IRAK1, AKT3, IL6, TLR4*
Ras signaling pathway	7/232	7.48e-4	0.0149	*EGFR, NFKB1, PLA2G4A, AKT3, KIT, PDGFRA, RHOA*
NOD-like receptor signaling pathway	6/178	0.00104	0.0177	*NFKB1, TRAF6, IL6, RHOA, TLR4, CASR*

Figure 1. Relative expression levels of miR-146b-5p in each group. Each dot represents a single sample.

HBV-HCC: HBV-related HCC, CHB: Chronic Hepatitis B

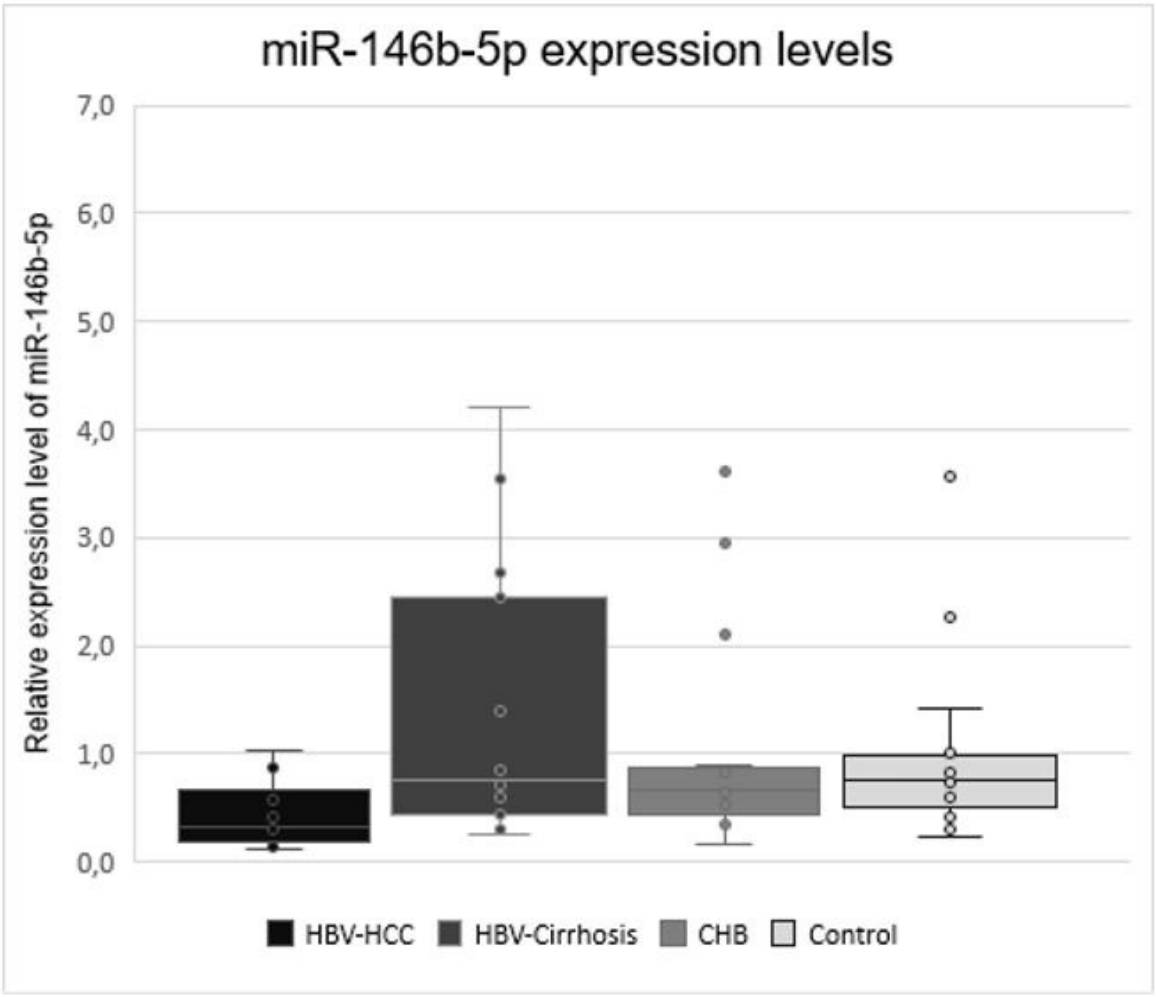

Figure 2. The potential molecular interactions between KEGG pathways and mRNAs target genes visualized by Network analysist. The nodes with a higher size and stronger color are consider as the most related pathway.