Research Article

In-vivo and *in-silico* miRNA analysis in HCC

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

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Abstract

Background and Aim: It is reported that miRNAs play an important role in hepatocellular carcinogenesis and may serve as non-invasive biomarkers for hepatocellular carcinoma (HCC). MiR-4510 and miR-146b-5p expression levels have been found to be associated with HCC. However, their associations with hepatitis B virus (HBV)-related HCC (HBV-HCC) are 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.

Materials and Methods: This cross-sectional study used the 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 was measured by qRT-PCR, calculated using the $2^{-\Delta\Delta Ct}$ methods. MIENTURNET was used to predict miRNA-target gene interactions. The Network Analyst was used to build protein-protein interactions.

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 the 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: Biomarker; cancer; HBV; hepatocellular carcinoma; miRNA.

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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] Additionally, there is a group of HBV-infected cases without cirrhosis who develop HCC.[5] In both cases, a 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 the early detection of HCC remains a major challenge.[6]

Diagnosing and determining the prognosis of HCC has been established 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 the capability of predicting the prognosis.[7] Numerous non-invasive biomarkers have been investigated for their potential role in increasing or surpassing the diagnostic and prognostic utility of AFP for HCC. Recently, miRNAs, which regulate gene expression at 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 that 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.[9,10] Additionally, accumulating evidence indicates that miRNAs can act as oncogenic or tumor suppressive factors involved in HCC progression.[11,12]

It is reported that down-regulation of miR-146b-5p in HCC tissues is related to malignant clinical features and poor prognosis. Using *in vitro* and *in vivo* studies, miR-146b-5p has been demonstrated as a novel inhibitor for tumor growth and metastasis in HCC. The multiple anti-cancer functions of miR-146b-5p are due to the inhibition of the TRAF6/p-Akt pathway. Researchers have 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 suggests it can be a novel biomarker for HCC. It has been demonstrated that miR-4510 may act 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, acting as a tumor suppressor in the liver by inhibiting tumoral cell growth and proliferation.[14] In HCC, the other molecular targets of miR-4510 are GPC3 and RAF1, subsequently controlling key biological and signaling pathways, including Wnt and RAS/RAF/MEK/ERK signals.[15] Moreover, its tumor suppressor potential has also been 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, miR-146b-5p and miR-4510 have been proposed as two promising biomarkers with the potential for utilization in the diagnosis of HCC.

The purpose of this study is to investigate the predictive value of miR-146b-5p and miR-4510 by detecting their serum expression levels in 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 genes 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

Serum samples were collected from 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 Marmara University, Institute of Helath Sciences. The patients' clinical, laboratory, and tumoral characteristics were extracted from the prospectively collected database for Marmara 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 of relationship to the experimental group. Each control was pair-matched by sex and age (±3 years). All participants were of Turkish ancestry. The participant recruitment was approved by the Committee for Ethics of Medical Experiments on Human Subjects, the Faculty of Medicine at Marmara 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 the biobank were centrifuged at 12,000 rpm for 15 minutes at 4°C. 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 were examined for miR-146b-5p and miR-4510 expression profiles to provide potentially important non-invasive biomarkers for the early diagnosis of HCC.

Total RNA Extraction from Serum

Total RNA was isolated using the 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 the Applied Biosystem BioRad C1000 Touch Thermal Cycler device. Cycler conditions were as follows: reverse transcription for 60 minutes at 42°C and inactivation for 5 minutes at 95°C.

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

Data Collection and Prediction of Target Genes of miR-146b-5p

Target genes of miR-146b-5p were retrieved from the miRTargetLink Human databases (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2), which provide miRNA-gene interactions using miRCarta, miRPathDB, miRbase, miRNA Tissue Atlas, and 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 miRNA-target interactions and perform network-based analyses.[18] 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. The Imex interactome database was selected to construct the PPI network via generic PPI in the 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 networks (hub genes). Eventually, the function of the hub genes was identified by KEGG mapper.

In the present study, we performed RT-PCR to evaluate miRNA expression levels and a bioinformatics analysis on the miRNA/target gene enrichment in 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 pathways was inves-

Table 1. Baseline characteristics and clinicopathological parameters

	HBV-HCC (n=15)	HBV- cirrhosis (n=15)	CHB (n=16)	Control (n=16)	p
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**

HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; CHB: Hepatitis B; MELD: Model for end-stage liver disease.

tigated. KEGG pathway analysis was 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 pathways in cancer. Based on the experimental certificate miRNA-target gene databases mentioned earlier, the miRNA-target gene regulatory network was constructed using the Network Analyst web tool.

Statistical Analysis

The median and interquartile range were used to display continuous skewed data, and the mean standard deviation was used if normally distributed. The results of categorical data are given as absolute numbers with percentages. For the comparison of continuous variables between four groups, the Kruskal-Wallis test was used when the data conformed to an abnormal distribution, and the Mann-Whitney U test was used for post-hoc analysis between two groups. The Chi-square test was used to compare categorical parameters. Logistic regression analysis was used to investigate the potential association of miR-146b-5p with CHB presence and progression. Spearman'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. Statistical significance was defined as $p<0.05$. All statistical analyses were conducted using 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 for 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 similar among all groups as well as body-mass index levels, presence of diabetes, and smoking. The HBV-HCC patients had a higher median Child-Pugh score compared to the HBV-cirrhosis group (7 vs. 5, p=0.031), while the median Model for End-Stage Liver Disease scores were not statistically different. Complications of cirrhosis, including ascites, esophageal varices, history of variceal bleeding, and hepatic encephalopathy, were not different among HBV-cirrhosis and HBV-HCC groups. As expected, the median alpha-fetoprotein (AFP) levels were higher in the HBV-HCC group.

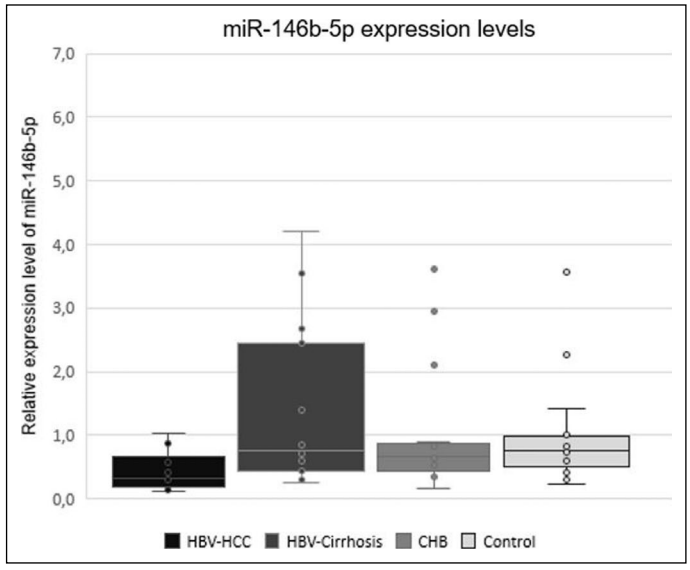

Figure 1. Relative expression levels of miR-146b-5p in each group. Each dot represents a single sample.

HBV-HCC: HBV-related HCC; CHB: Chronic hepatitis B.

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 the housekeeping gene SNORA66. The quantitative expression values ($2^{-\Delta\Delta Ct}$ values) of all groups are shown in Figure 1. It was observed that ΔCt values in the groups

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
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. HBB: Hepatitis B virus; HCC: Hepatocellular carcinoma; HBV-HCC: HBV-related HCC; CHB: Chronic hepatitis B; n: Sample size.

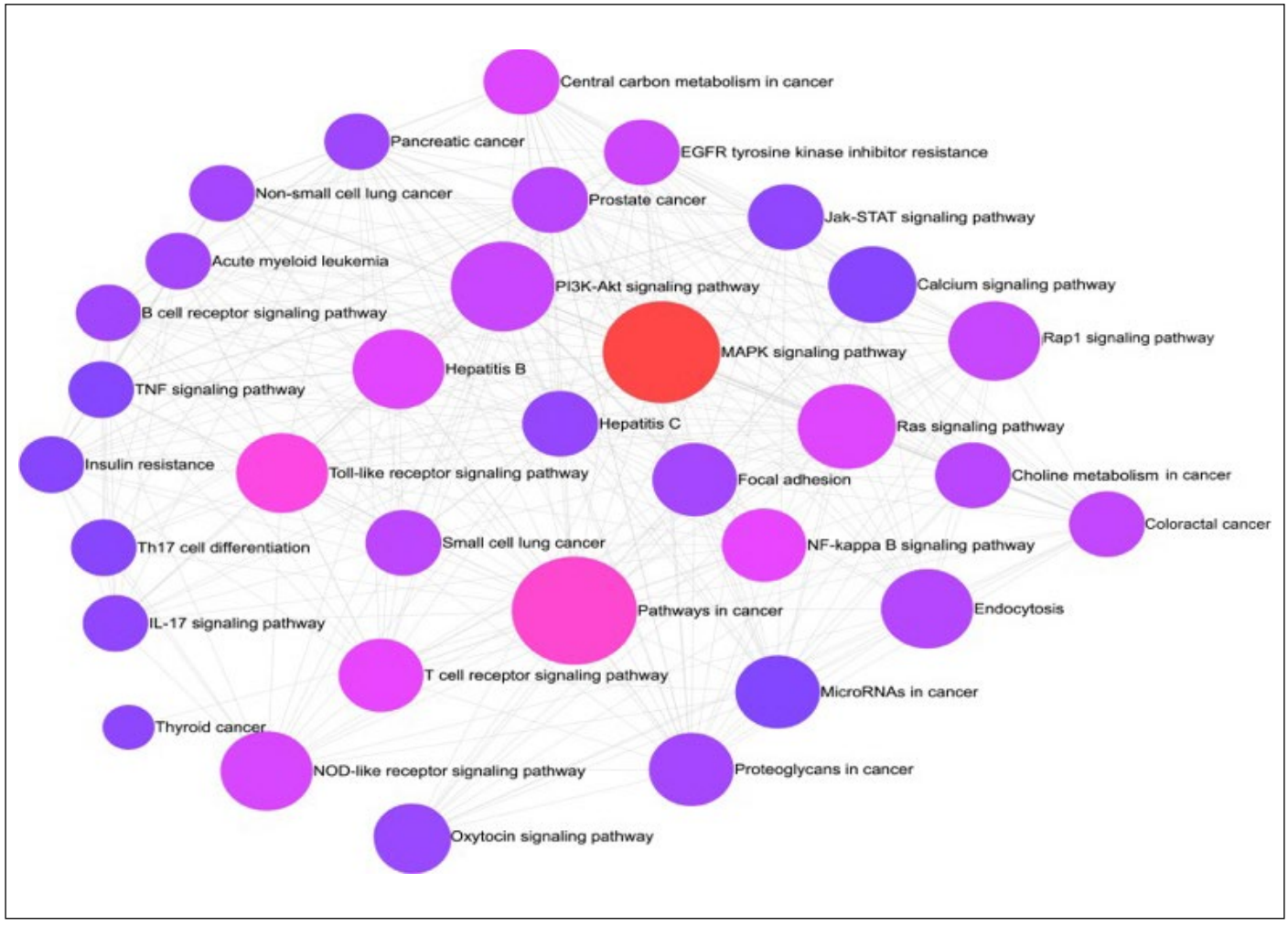

Figure 2. The potential molecular interactions between KEGG pathways and mRNAs target genes visualized by Network analysist. The nodes with a higher size and stronger color are consider as the most related pathway.

were not normally distributed. For this reason, the difference was examined with the Kruskal-Wallis test. Expression levels of miR-146b-5p in serum were found to be significantly lower in the HBV-HCC group compared to the HBV-cirrhosis, CHB, and healthy control groups. The expression levels of miR-146b-5p and their comparison among groups are shown in Table 2. A significant difference was found between the groups regarding miR-146b-5p levels (p=0.009) (Table 2). Significant differences were also found when the HBV-HCC group was compared with the HBV-cirrhosis group (p=0.006) and the healthy control group (p=0.005) (Table 3). We further examined the correlation between the expression of serum miR-146b-5p and clinical parameters. No significant association was found between miR-146b-5p and clinicopathological parameters (all p values >0.05). As for miR-4510, expression was

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

	Mean rank difference	p
HBV-HCC vs. HBV-cirrhosis	-18.233	**0.006**
HBV-HCC vs. CHB	-14.719	0.059
HBV-HCC vs. control	-19.357	**0.005**
HBV-cirrhosis vs. CHB	3.515	0.476
HBV-cirrhosis vs. Control	-1.124	0.793
CHB vs. control	-4.638	0.454

HBB: Hepatitis B virus; HCC: Hepatocellular carcinoma; 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	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

HCC: Hepatocellular carcinoma; KEGG: Kyoto Encyclopedia of Genes and Genomes;

observed in only three individuals in the control group by RT-qPCR, thus it was not included in further analytic studies. miR-4510 was not expressed in the 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 that 120 genes were targets of miR-146b-5p, and Table 4 represents 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 the miRTargetLink Human database as described earlier. The 120 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 a $P<0.05$ value for KEGG pathway enrichment analysis of miR-146b-5p; "MAPK signaling pathway" and "Pathway in cancer" were the most statistically significant pathways (Fig. 2). Ten significant HCC-related pathways are listed in Table 5.

Discussion

It is well known that miRNAs play various roles in different cancer types, including HCC.[20,21] However, the potential of miRNAs as biomarkers for the detection of HCC is still underexplored. In the literature, the anti-cancer functions of miR-146b-5p and miR-4510 in HCC and other cancer types have been shown. Therefore, it can be speculated that these miRNAs may be novel promising biomarkers for the prediction of HCC.[22] Moreover, there are no sufficient in-vivo studies investigating the association between these miRNAs and HBV-related HCC, despite chronic HBV patients being 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 to HBV infection. Another interesting result is that miR-4510 cannot be detected in comparable levels in the serum 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 investigated further. 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 has been conducted.[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, making 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, 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 glypican-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.[16] It can also reduce the growth, migration, and invasion of GIST-882 cells. 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 TCGA-LIHC tumor tissue in comparison to normal liver tissues in the 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 TCGA-LIHC tumor tissue and normal tissues ($p>0.05$).

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 the "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 the specific population (Turkish), 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 act 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. 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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