# Bioinformatics Analysis IdentifiesThrombospondin-1 and its related micro RNAs as biomarkers for Hepatocellular carcinoma

**Wei xuan** ( xuanwei@jlu.edu.cn )
China-Japan Union Hospital of Jilin University
**Dandan Song**
Jilin University
**Yunjia Ren**
Jilin University
**Ming Li**
Jilin University

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# Abstract

Hepatocellular carcinoma (HCC) has a poor prognosis, while the diagnosis biomarkers or treatment targets for HCC are still lacking. Recently, mining strategies on public access databases has been successfully used to discover novel and sensitive biomarkers. In this study, bioinformatics analysis of the genomic and transcriptome data of HCC PBMC samples obtained from the Gene Expression Omnibus datasets. The analysis results showed that thrombospondin-1 (THBS-1) was dysregulated in HCC, and might be as biomarkers as it is a secretory protein and found to participate in liver dysfunction including HCC development. THBS-1 related micro RNAs are identified by bioinformatics analysis and tested as biomarkers as they are altered in many tumors and stable in circulation. The results showed that plasma THBS-1 and miR-194 were down-regulated in HCC patients and their correlations were positive. Whereas plasma level of miR-338-3p was significantly increased in the HCC patients and negatively correlated with THBS-1 and they showed a highly significant diagnostic value in discriminating HCC. In addition, THBS-1 and miR-338-3p combination displayed higher predictive power than them alone, and addition of miR-338-3p might enhance predictive power of AFP for HCC. Our data showed that THBS-1 and miR-338-3p displayed a highly significant diagnostic value in discriminating between HCC patients and control subjects. Addition of miR-338-3p might enhance the predictive potency of AFP for HCC.


# Introduction

Hepatocellular carcinoma (HCC) is well known as the sixth most common malignant tumor with poor prognosis and causes the third cancer death worldwide [1]. The 5-year survival rate of HCC is less than 40%, the poor prognosis partly due to the lack of diagnostic marker [2]. Clinically, α-fetoprotein (AFP) is widely used as diagnosis biomarker for HCC, but it is negative in 30–40% of HCC patients and positive in some subjects with chronic hepatitis or liver cirrhosis [3]. Therefore, it is important to identify promising molecular biomarkers, especially circulating biomarkers, or targets for HCC [4, 5].

Data mining strategies on public access databases has been successfully utilized to discover novel and sensitive biomarkers [6]. Gene Expression Omnibus (GEO), an available open access database, provide abundant microarray resources for searching deregulated genes of HCC. Bioinformatics analysis of significant deregulated genes of HCC PBMC samples obtained from the GEO showed that thrombospondin-1 (THBS-1), a matricellular glycoprotein, was significantly changed and reported to participate in liver dysfunction or angiogenesis of HCC [7, 8, 11]. Consistently, low expression of THBS-1 in HCC tissues is reportedly correlated with a poor prognosis [9, 10]. In addition, microRNAs (miRNAs) are small non-coding RNA molecules that can regulate gene expression in many biological processes including tumor genesis [12]. And, miRNAs are altered in many tumors including HCC and their remarkable stability in blood, therapy could be as biomarkers [13]. Circulating micro RNAs such as miR-21 or miR-122 have been investigated for HCC biomarkers [14, 15]. Therefore, circulating THBS-1 related micro RNAs might be as biomarker for HCC.

In this study, we found that THBS-1 was significantly changed in HCC PBMC samples by Whole-genome DNA microarray analysis and predicted THBS-1 related micro RNA using the publicly available TargetScan. We further tested the potency of THBS-1 and its related micro RNAs as the biomarkers for HCC.

## Methods

# Patients

From January 2018 to August 2018, 67 patients were newly diagnosed with HCC at China-Japan Union Hospital of Jilin University, Changchun, China, were consecutively enrolled in this study. The diagnosis of HCC based on CT and/or magnetic resonance imaging (MRI) observations as well as AFP assay. In addition, 47 matched subjects without cancers were used as control. All blood samples (5 ml per patient) were collected before those received any treatments via a direct venous puncture and placed into tubes containing sodium citrate, centrifuged at 1000 × *g* for 5 min and 3000 × *g* for 10 min, the layer of the supernatant (plasma) was carefully transferred into other tubes and stored at − 80 °C. Written consent was obtained from all subjects, and the study protocol was approved by the ethics committee of Jilin University (2018082107).

# GEO analysis of gene expression in HCC samples

Microarray data of GDS4882 including 10 patients with HCC and 10 normal subjects were obtained from the Gene Expression Omnibus database (https://www.ncbi.xilesou.top/gds/?term=GDS4882). The Gene Ontology (GO) terms of differential proteins were analyzed using the online OmicsBean bioinformatics resource (http://www.omicsbean.cn), which is a multi-omics data analysis tool that can be applied to dynamic results [16].

# THBS-1 related miRNAs prediction

miRNAs targeting THBS-1 was first predicted using the publicly available TargetScan (http://www.targetscan.org), and then confirmed by miRBase (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5).

# Assay for plasma THBS-1, miR-194 and miR-338-3p

We tested the plasma level of THBS-1 using the ELISA kit from Elabscience, following the manufacturer's instructions. Absorbance was measured at 450 nm (primary wave length).

MiRNAs were extracted from the plasma samples contained 50 pmol/L Caenorhabditis elegans miR-39 (cel-miR-39), which was used as an external reference, following the instruction of miRcute miRNA Isolation kit (TRANS GEN, Beijing, China). Each sample was eluted in 100 μL of RNAse-free water. qPCR assay Poly-(A) tailing and reverse transcription were performed with the miScript reverse transcription kit (TRANS GEN, Beijing, China). miRNAs were quantified using a miRNA quantitative reverse transcriptase-polymerase chain reaction assay as per the manufacturer's instructions. The PCR was performed as

follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Relative quantification was performed using the ViiA 7 Software v1.1 (Applied Biosystems). The micro RNA assay primers used were miR-194 forward: 5'-TAACAGCAACTCCATGTGGA-3', miR-338-3p 5'-TCCAGCATCAGTGATTTTGTTG-3' and cel-miR-39 forward: 5'-TCACCGGGUGUAAATCAGCTTG-3'. Negative controls using nuclease-free water were included with every real-time PCR operation and cycle threshold (CT) values > 35 viewed as negative. All samples for miRs were run in one assay and all reactions were run in triplicate. Analysis of relative gene expression levels was performed using the formula 2-ΔCT with ΔCT = CT (target gene)-CT (control).

# Statistical analyses

SPSS 26.0 for Windows v. was used to perform the statistical analyses. Data are presented as the mean ± SD and median for the general characteristics of the subjects. Differences of means between HCC and control groups were assessed using Two-tailed t-tests. Values with a $p < 0.05$ were considered to indicate statistical significance. The relationships between plasma miR-194 and miR-338-3p with THBS-1 were assessed using the Spearman correlation test. Binary logistical regression analysis was also used to evaluate the predictive powers of plasma THBS-1, miR-194, and miR-338-3p for HCC. As the validation cohort is relatively small, bootstrap analysis of 1000 iterations was used to correct the false positive findings. The SPSS 26.0 version of Receiver operating characteristic (ROC) analysis containing the ROC curves and overall model quality was used to evaluate the individual predictive accuracy of the candidate biomarkers or their combinations.

# Results

### Baseline characteristics

67 patients with HCC and 47 matched control subjects without HCC were enrolled. Their clinical characteristics and biochemical parameters are listed in Table 1. Age; sex; hypertension; smoking history; drinking history; Hepatitis history (Hepatitis B and Hepatitis C) were not different between HCC and control groups. In addition, the level of AFP in 31.13% of HCC patients was lower than 20 ng/ml, but it was detected in subjects without cancers.

### Analysis of gene or micro RNA expressions of HCC

Microarray data of GDS4882 including 10 patients with HCC and 10 normal subjects were obtained from the Gene Expression Omnibus database. Genes of HCC samples were analyzed using the online OmicsBean bioinformatics resource (http://www.omicsbean.cn), which is a multi-omics data analysis tool that can be applied to dynamic results. The results showed that 64 genes were significantly up-regulated and 5 genes were down-regulated with over 1.5 fold change ($p < 0.01$) (Figure.1A and table. 1S). Among them, THBS-1 attracts more attention as it has been found to participate in liver dysfunction and HCC development.

We further assayed THBS-1 related microRNAs by the TargetScan database and found that there was the only miR-338 that could bind to converse and no-converse sites located with the 3’UTR of THBS-1 mRNA

(nt 38–44, 559–665, respectively) and with a high target score (Figure.1B). In addition, miR-194 was changed in HCC and promoted angiogenesis by inhibiting THBS-1, indicating that miR-194 might be used as a biomarker or therapeutic target for HCC [17].

### The plasma levels of THBS-1, miR-194, and miR-338-3p

To explore the biomarkers for HCC, we tested plasma levels of THBS-1 and miR-194, miR-338-3p, which was reported or predicted to target THBS-1, respectively. The results showed that plasma THBS-1 was significantly lower in HCC patients, compared with control subjects (765.96 ± 612.28 vs 265.49 ± 163.99 pg/ml, $p < 0.01$) (Figure.2A), The plasma miR-194 was decreased, while the plasma miR-338-3p was increased in HCC patients compared with control subjects (0.36 ± 0.22 vs 1.00 ± 0.70 fold, 3.46 ± 4.24 vs 1.00 ± 0.89 fold, $p < 0.05$, $p < 0.05$, respectively) (Figure.2B and Figure.2C). Bootstrap analysis with 1000 iterations showed that there was significance of THBS-1 between two groups, whereas there were no significances of miR-194, miR-338-3p between groups ($p = 0.38$, $p = 0.17$ respectively).

### The correlations of THBS-1 and miR-194, miR-338-3p in patients with HCC

MiR-194 and miR-338-3p were reported or predicted to target THBS-1, respectively. Thus, we also investigated the correlations of THBS-1 with miR-194 or miR-338-3p. The results showed that plasma THBS-1 positively correlated with plasma miR-194 ($R = 0.23$, $p < 0.05$) (bootstrapped 95% CI:0.04, 0.28) (Figure.3A), but negatively correlated with miR-338-3p ($R= -0.21$, $p < 0.05$) (bootstrapped 95% CI: 0.00, 0.06) (Figure.3B).

### Comparison of the predictive powers of THBS-1, miR-194, and miR-338-3p for HCC

As plasma THBS-1, miR-194 or miR-338a were significantly changed in HCC patients, we evaluated the predictive powers of THBS-1, miR-194, miR-338-3p, and hepatitis history by binary logistical regression analysis (Table 2). The results showed that plasma THBS-1 (odds ratio: 1.00; 95% CI: 0.99, 1.00; $p < 0.01$) (bootstrapped 95% CI: 0.00, 0.06; $p < 0.01$), miR-338-3p (odds ratio: 1.77; 95% CI: 1.05, 3.89; $p < 0.05$) (bootstrapped 95% CI: 0.00, 0.06; $p < 0.05$), were significantly correlated with HCC status. However, there were no significance of miR-194 and Hepatitis between two groups. These results suggested that plasma THBS-1, miR-338-3p showed a highly significant diagnostic value in discriminating between HCC patients and control subjects. Notably, AFP as positive control was also evaluated and displayed a highly significant diagnostic value (odds ratio: 1.32; 95% CI: 1.04, 1.67; $p < 0.05$) (bootstrapped 95% CI: 0.11, 1.27; $p < 0.05$).

We next performed ROC analysis containing ROC curve and overall model quality to evaluate the predictive powers of plasma THBS-1, miR-338-3p, and AFP for HCC. ROC analysis (Figure.4A) showed that the area under the ROC curve (AUC) for 1/THBS-1 was 0.73 (95% CI: 0.66, 0.83; $p < 0.01$) and the optimal cut-off value was 328.28 pg/ml with sensitivity and specificity of 59.6 and 62.7%, respectively. The AUC for miR-338-3p was 0.72 (95% CI: 0.62, 0.81; $p < 0.01$ and the optimal cut-off value was 1.06 fold with sensitivity and specificity of 71.6 and 68.2%, respectively. The AUC for AFP was 0.92 (95% CI: 0.86,

0.97; $p < 0.01$) and AFP displayed higher predictive powers than that of 1/THBS-1 or miR-338-3p ($p < 0.01$, $p < 0.01$, respectively). Overall model quality displays the value of the lower bound of the confidence interval of the estimated AUC, and predictive model is considered good when the value is over 50%. As shown in figure.4B, the overall model quality in all data is over 50%.

We further tested whether the predictive powers of combination of 1/THBS-1 or miR-338 with AFP by ROC analysis. The resulted (figure.4C and figure.4D) showed that the AUC of 1/THBS-1 plus miR-338 was 0.82 (95% CI: 0.74–0.89; $p < 0.01$) and higher than that of 1/THBS-1 or miR-338 alone ($p < 0.05$, $p < 0.01$, respectively). And, the overall model quality of combination of miR-338 with 1/THBS-1 was higher than them alone (0.74 versus 0.62 or 0.63, respectively). The AUC of 1/THBS-1 plus AFP was 0.93 (95% CI: 0.74–0.89; $p < 0.01$) and the overall model quality of combination of 1/THBS-1 with AFP was 0.88, there was no statistical significance between combination of 1/THBS-1 with AFP than AFP alone. AUC of miR-338 plus AFP was 0.95 (95% CI: 0.91–0.99; $p < 0.01$), and higher than AFP alone, but there was no statistical significance. The overall model quality of combination of miR-338 or 1/THBS-1 with AFP were higher than AFP alone (0.91 versus 0.86).

# Discussion

THBS-1 is found to inhibit angiogenesis in many tumors including HCC and plays an important role in tumor growth, metastasis, and prognosis [8, 9]. The low THBS-1 expression in the tissue of HCC is negatively correlated with ephrin-A1 expression, which could induce the expression of the angiogenesis factors [10]. Conversely, another study showed that higher THBS-1 levels in HCC tissues were also found and positively correlated with VEGF levels, and were linked to poor survival in HCC patients [18]. It was also reported that there was no difference in THBS-1expression between normal and HCC liver samples and there was no positive correlation of THBS-1 expression levels with tumor grades and angiogenesis in HCC patients [19, 20]. In this study, we found that the gene of THBS-1 was decreased in the HCC PBMC samples; the protein level of THBS-1 was also decreased in the serum of HCC patients.

MiRNAs are altered in HCC and could be as the therapeutic targets or biomarkers [13]. The miRNAs targeting THBS-1 might be as biomarkers or therapeutic targets for HCC and also were investigated in this study. Many miRNAs targeting THBS-1 such as miR-17-92 and miR-194 have been identified. MiR-18a and miR-17-5p belonged to the miR-17-92 cluster and were up-regulated in the serum and tissues of HCC [21, 22, 23]. The high levels of miR-18a and miR-17-5p correlated with poor prognosis as they were found to inhibit the proliferation or metastasis of HCC, respectively [24, 25]. MiR-194, another micro RNA targeting THBS-1, was down-regulated in the majority of HCC tissues but not detected in the plasma or serum [26]. Our data also showed that plasma miR-194 was decreased in HCC patients. Consistently, miR-194 inhibited proliferation and metastasis in hepatocellular carcinoma and low miR-194 expression linked with poor prognostic significance [27]. Although miR-194 was reported to target THBS-1, our data showed that there was a positive correlation of miR-194 with THBS-1 in the plasma of HCC patients. The concentrations of miRNAs in plasma may be affected by multiple parameters such as tissue expression

and processing, the release, and stability [28]. For example, the plasma miR-126 was contrasted with it in ECs during ACS [28]. In addition, microRNAs might be consummated by some circulating lnc RNAs [29].

In this study, miR-338-3p was predicted to target THBS-1 and our data also showed that the high level of plasma miR-338-3p negatively correlated with THBS-1. Consistently, the miR-338-5p was also increased in plasma of HCC patients [30]. However, the direct relationship of miR-338-3p with THBS-1 was not reported. In addition, the level of miR-338 in plasma might be different from that in the tissue. MiR-338-3p was decreased in HCC tissues and low miR-338-3p could promote the proliferation, metastasis, and angiogenesis of HCC and correlated poor prognosis [31, 32]. Therefore, the level of miR-338-3p in different tissues, as well as its direct relationship, needed further study.

AFP is widely used for HCC diagnosis, but it is negative in 30–40% of HCC patients. In this study, we also found that AFP value in 31.13% of HCC patients was lower than 20 ng/ml. In this study, we also evaluate the predictive powers of miR-194, miR-338-3p and THBS-1 for patients with HCC using ROC and binary logistical regression analysis. These results suggested that plasma THBS-1, miR-338-3p showed a highly significant diagnostic value in discriminating between HCC and control subjects. And, combination of THBS-1 and miR-338-3p displayed higher predictive power for HCC than them alone. Addition of miR-338-3p might enhance the predictive potency of AFP for HCC.

# Conclusion

The level of THBS-1 was conversely correlated with miR-338-3p in the plasma of HCC patients and they alone or combination showed a highly significant diagnostic value in discriminating between HCC patients and control subjects. Addition of miR-338-3p might enhance the predictive potency of AFP for HCC.

# Limitations

In this study, we investigated the levels and predictive potencies of THBS-1 and its related micro RNAs in HCC, but the sample size was small and analyzed from a single center, larger studies are needed to confirm the current results. Most of HCC cases develop from cirrhosis, we should provide results in a consistent group of patients with cirrhosis without HCC to be compared with those with HCC. However, persistent infections by Hepatitis B or C are main risk factors for cirrhosis and HCC, therefore the matched control subjects with hepatitis history were enrolled in this study.

# Declarations


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### Conflict of Interest

The authors have no conflicting financial interests.

### Availability of data and materials

Data is available upon request.

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# Tables

**Table.1 Clinical characteristics and biochemical parameters of the patients**

| Variable | Control | HCC | P value |
|---|---|---|---|
| | n = 47 | n = 67 | |
| age(y) | 57.68 ± 9.88 | 55.59 ± 11.01 | 0.301 |
| Male n (%) | 22(46.80) | 36(53.73) | 0.472 |
| Somking n (%) | 20(42.55) | 26(38.8) | 0.687 |
| Diabetes n(%) | 8(17.02) | 18(26.86) | 0.223 |
| Hypertension, n (%) | 7(14.89) | 19(28.35) | 0.092 |
| Hepatitis history n (%) | 23 (48.94) | 42 (62.69) | 0.13 |
| AFP (ng/ml) | 3.34 ± 2.38 | 232.63 ± 277.28 | 0.01 |

**Table 2.Binary logistical regression analysis of TSP-1, miR-194, miR-338-3p, Hepatitis, in patients with HCC**

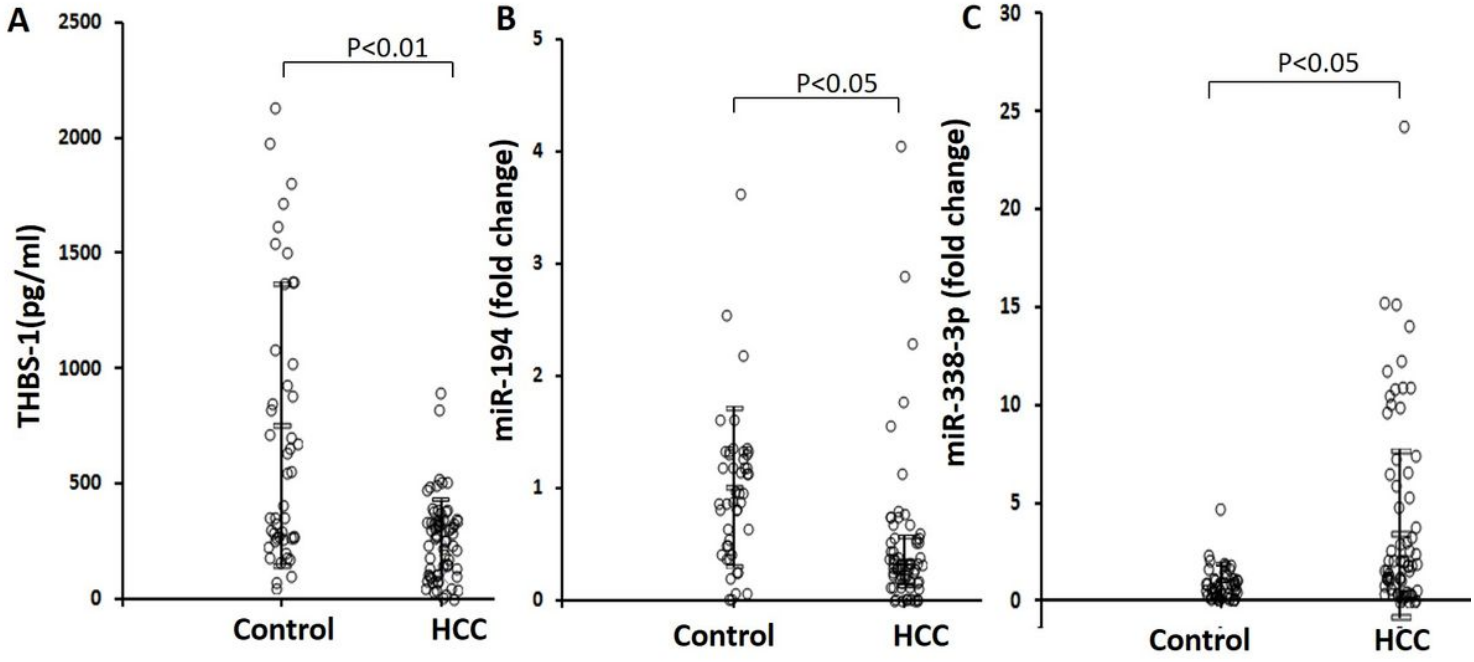
| Variable | p-value | OR | 95% CI | | bootstrap | bootstrap 95% CI | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | p-value | Lower | Upper |
| THBS-1 | < 0.01 | 1.00 | 0.99 | 1.00 | < 0.01 | -0.12 | -0.02 |
| miR-194 | 0.27 | 1.29 | 0.76 | 2.21 | 0.27 | -2.34 | 2.34 |
| miR338-3p | 0.01 | 2.02 | 1.05 | 3.89 | 0.01 | 0.30 | 2.18 |
| Hepatitis | 0.11 | 0.26 | 0.05 | 1.38 | 0.10 | -7.89 | 0.25 |
| AFP | 0.04 | 1.32 | 1.04 | 1.67 | 0.04 | 0.11 | 1.27 |

Bootstrap significance or 95% CI is two-tailed with 1000 iterations

# Figures

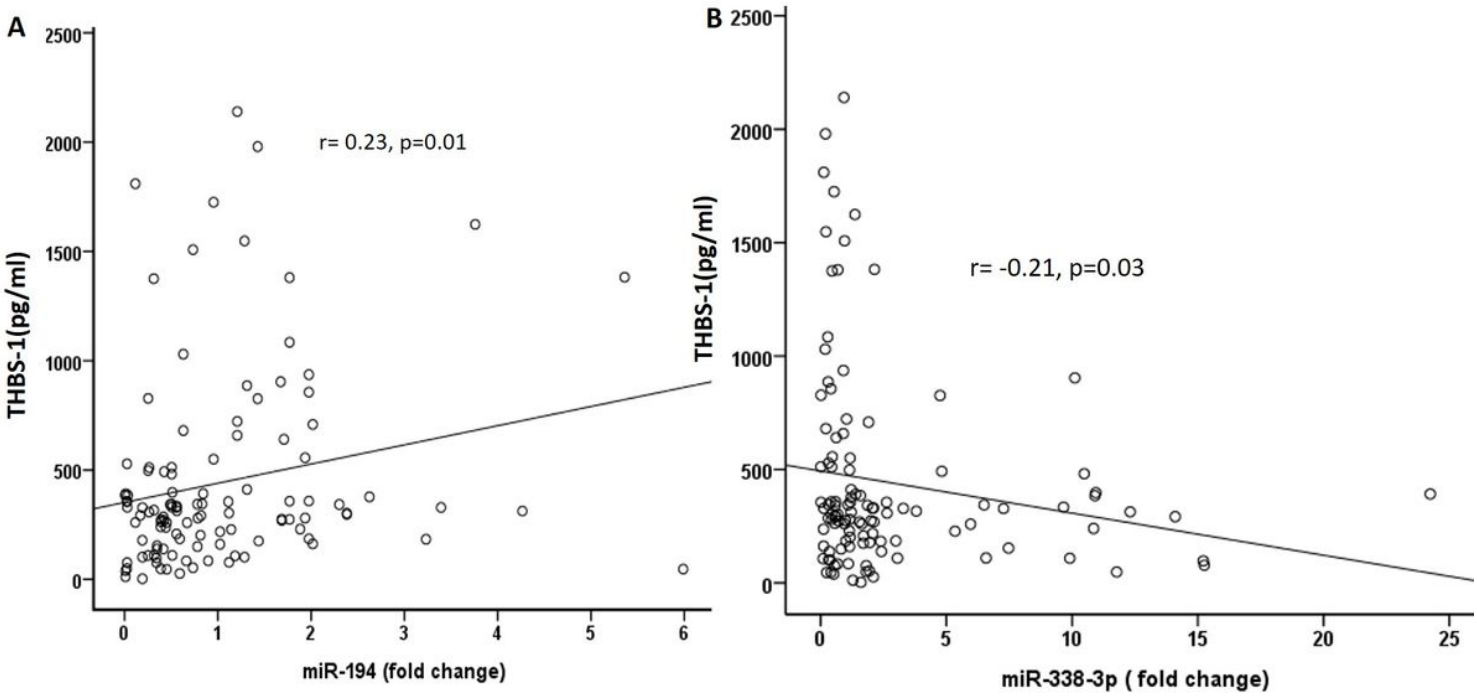

## Figure 1

Analysis of gene or micro RNA expression in HCC samples. A GEO analysis of gene expression in HCC PBMC samples, B Analysis of THBS-1 related micro RNA expressions using the publicly available TargetScan.




## Figure 2

The plasma levels of THBS-1 and miR-194, and miR-338-3p. A. The plasma level of THBS-1 in HCC patients or subjects without cancers, B. T The plasma level of miR-194 in HCC patients or subjects without cancers, C. The plasma level of miR-338-3p in HCC patients or subjects without cancers.




## Figure 3

The correlations of THBS1 with miR-194, and miR-338-3p. A. The correlation of THBS1with miR-194, B. The correlation of THBS1 with miR-338-3p.

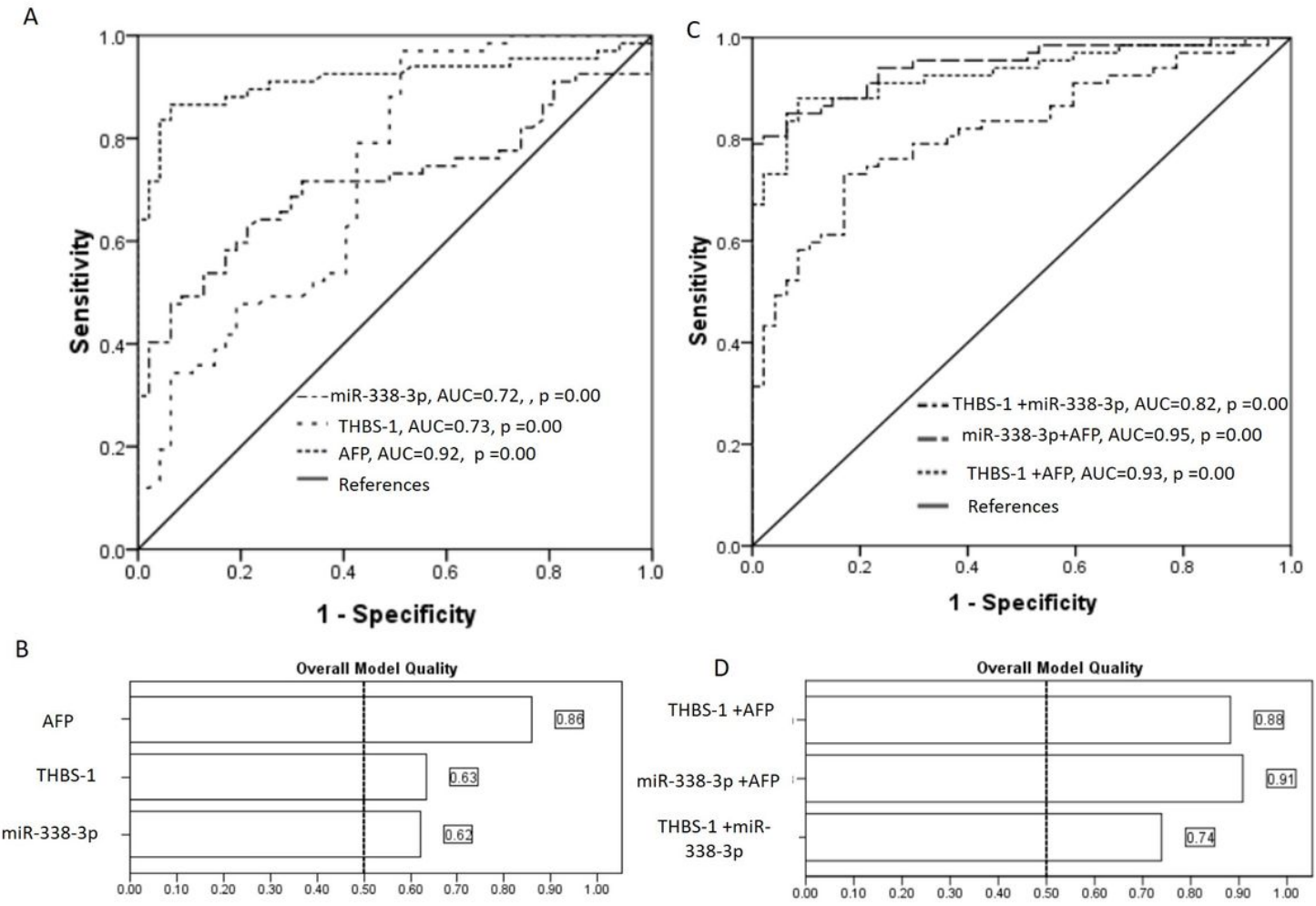



**Figure 4**

The ROC analysis of the predictive power of THBS1, miR-338-3p, and AFP for HCC status. A. ROC curve of 1/ THBS1, miR-338-3p, and AFP for HCC, B. Overall model quality of 1/ THBS1, miR-338-3p, and AFP for HCC C. The ROC curve of 1/THBS1, miR-338-3p, and AFP combination for HCC, D. Overall model quality of 1/THBS1, miR-338-3p, and AFP combination for HCC

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