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Identification of Novel Cancer Biomarkers in LIHC

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Keywords: Liver Hepatocellular Carcinom, miR-9-5p, miR-452-5p, PDK4, COLEC10

Abstract: LIHC (Liver Hepatocellular Carcinoma) is the most common malignant cancer around world. Recent studies reported that miRNA plays vital roles in tumor development and progression, suggesting the miRNA in LIHC could been regarded as potential prognostic biomarkers. Assessment of the up-regulated miRNA and its target protein as novel cancer biomarkers in LIHC cancer cells. First, we assessed the up-regulated and down-regulated miRNA in LIHC and normal tissue from The Cancer Genome Atlas (TCGA) database. Next, we performed the survival plot and time ROC plot to select the LIHC related miRNA. Then, by introduction of TargetScan Human software and TCGA database, we got the miRNA related proteins. Lastly, by series of cell biology and biochemistry assays, we illustrated miRNA targeted proteins could affect cancer cell physiological function. Identification of miR-10b-5p, miR-224-5-p, miR-183-5p, miR-182-5p, miR-217, miR-196b-5p, miR-452-5p and miR-9-5p are overexpressed in LIHC than in normal tissue. The elevated expression of miR-452-5p and miR-9-5p are associated with significantly poorer over survival of LIHC patients, miR-9-5p and miR-452-5p could restrict the expression of PDK4 and COLEC10 respectively, down-regulated level of PDK4 and COLEC10 are correlated with poorer over survival in LIHC patients. Moreover, overexpression of PDK4 and COLEC10 in LIHC cancer cells generated cell apoptosis and growth retardation, cell derived xenograft assay found the PDK4 and COLEC10 brought the development and progression retardation of LIHC. GO and KEGG analysis showed that PDK4 and COLEC10 positive correlation genes are involved in PPAR signaling pathway and PI3K−Akt signaling pathway. We found two new diagnostic markers in LIHC, miR-9-5p and miR-452-5p, which may facilitate the early diagnosis and treatment, we also uncovered that PDK4 and COLEC10 could been identified as tumor suppressors by misregulation of cell psychological function. In LIHC cancer cell, miR-9-5p and miR-452-5p acted as oncogene factors by directly suppress the expression of PDK4 and COLEC10.Activation of PPAR signaling pathway and PI3K-Akt signaling pathway may be viewed as potential therapeutic approach in LIHC.

1. Introduction

Primary liver cancer is a global health crisis and its occurrence is growing around the world [17].

Liver hepatocellular carcinoma (LIHC), account for 80~90% of all primary liver cancer cases, is one of most prevalent and aggressive human malignancies worldwide [23]. The development and progression of LIHC is a multistage and multifactor consequence, including infection of hepatitis B virus and hepatitis C virus, alcohol abuse, diabetes, obesity and unhealthy living habits [2][15]. Although great achievements and advances in early diagnosis and prognosis, it's still estimated that over 1 million cases of LIHC by 2025 [7]. Even if there are various of therapeutic approaches of LIHC, such as tumor excision, liver transplantation, chemotherapy, radiotherapy and immunotherapy, the high risk of recurrence and metastasis is challenging subjects [9].

Micro RNAs (miRNA; miR) is a large classification of functional short non-coding RNAs that have been identified as regulator of genes expression in post-transcription period [5][22].miRNA negatively regulate gene expression by directly binding the untranslated regions (UTRs) of target miRNAs, resulting in mRNA degradation or the blockade of miRNA translation [19].

Previous studies reported that over 30% of protein-code genes are involved in regulation of miRNA, increasing number of evidences show that miRNA participate in multiple biological processes. Especially, in the last decades, their importance in tumorigenesis has been illustrated [14][12].In human cancer, the expression of miRNA is dysregulated through various mechanism, dysregulated miRNA function as oncogenes or tumor suppressors in tumor development and progression, and what's more, accumulating experimental suggests that misregulation of miRNA in tumor have been shown to affect the cancer hallmarks, indicating miRNA and their target proteins have been emerged as novel cancer diagnosis, prognosis and therapeutic targets or tools [1][20].

Most recently some specific miRNA were found to be associated with the clinical pathological features of LIHC, such as metastasis, recurrence, and prognosis, demonstrating that miRNA and their target proteins are essential for LIHC progression [11].

In this study, bioinformatics method are performed to screen the differentially expressed miRNAs (DEMs), cell biology and biochemistry assays are implemented to further investigate their physiological functions.

2. Materials and methods

2.1 Data Acquisition

All clinical data were obtained from The Cancer Genome Atlas (TCGA) database,LIHC cancer cell line HepG2 purchased from ATCC(American Type Culture Collection). All cells were grown in DMEM supplemented with 10% fetal bovine serum(FBS) and cultured at 37℃with 5% CO2.

2.2 Antibodies

The following antibodies were used: PDK4 (Abclonal Rabbit pAb A13337), COLEC10 (CST Rabbit mAb #77180).

2.3 RNA extraction and Real-time PCR

Total RNA was extracted from 6cm dish cell lines by Trizol reagent(Invitrogen), according to the product's protocol, Gene relative expression level was determined by ABI 7500 Fast system, real-time primer Gapdh: F 5'-GCCTGGAGAAACCTGCCAAG-3', R 5'-CGGCATCGAAGGTGGAAGAG-3'. PDK4: F1 5'-ggagcatttctcgcgctaca-3', R1 5'-tccatcaggctctgtatata-3', F2 5'-Atactccactgcaccaacgc-3', R2 5'-gcctcagagctcatctgataa-3'. COLEC10: F1 5'-gtgaatgaccttgaaagggagg-3', R1 5'-atggtaagatggcactctgt-3'. F2 5'-atggggccgaaaggaattaa-3',R2 5'-tgtcttgagccgagcaatacta-3'.

2.4 Cell apoptosis test

Cell apoptosis were analyzed by Annexin V-FITC/PI detection kit (Vazyme#A211). In briefly, collected and rinsed cell with PBS buffer, subsequently, cell were incubated with V-FITC and PI staining solution in the dark at room temperature (20℃ ~ 25℃) for 10 min, add binding buffer and mix gently. The samples were detected by flow cytometry within 1 h after staining.

2.5 Cell proliferation test

HepG2 cell lines were seeded into 96 well dish and treated with the indicated agent CCK-8(Beyotime#C0037). Cell proliferation was measured by microplate reader at 450 nm, The operation was followed by manufacturer's protocol. Generally, cell was divided into several groups, add agent at 0 hours, measured the OD 450nm at 24, 48, 72 , 96, 120, and 144 hours respectively, collected the data into flow cytometry.

2.6 Cell cycle Analysis

The cell cycle was detected by flow cytometry with propidium iodide (PI) staining (Beyotime#ST511). After drug treatment, cell was collected and fixed into 70% ethanol at 4℃ overnight, next, cells were rinsed with PBS twice and labeled into PI agent in dark, cell than measured by cytometry.

2.7 Dual luciferase reporter assay

The dual luciferase reporter assay was conducted to determine whether miR-9-5p and miR-452-5p directly bind to PDK4 and COLEC10, respectively. Cells in a 24-well plate were co-transfected with either mimics or inhibitors of miR-9-5p and miR-452-5p, along with luciferase reporter plasmids containing either wild-type (wt) PDK4 and COLEC10 or their respective mutations. After 48 hours, the luciferase signal intensity was measured using the Dual Luciferase Reporter Assay Kit (Vazyme #DL101), with Renilla luciferase serving as an internal reference.

2.8 Identification of DEMs in LIHC

Tumor specimens and normal tissues miRNA-seq data were downloaded from TCGA database, the miRNA expression were respectively divided into high and low groups based on the DESeq2 workflow with the standard function: $|LogFC| \geq 1.5$ and $P < 0.05$. In total, 8 DEMs were identified.

2.9 Overall Survival plot

Implementation of Kaplan-Meier (K-M) analysis and Univariate Cox Regression Analysis in data from TCGA.

2.10 ROC(receiver operating characteristic curve) plot

I implemented the pROC package in R to organize the downloaded data. For visualization, I used the ggplot2 package in R to create graphical representations of the data.

3. Results

3.1 Identification of up-regulated and clinical significant miRNAs in LIHC

Previous researches reported the aberrant expression of miRNAs in tumor usually linked to cell physiological functions, indicating the elevated expression miRNAs is crucial for cancer development. In order to investigate the functional miRNA in LIHC, we firstly download the LIHC data in TCGA, by introduction of bioinformatics analysis, the volcano plot identified up-regulated and down-regulated miRNAs in LIHC, miR-10b-5p, miR-224-5p, miR-183-5p, miR-182-5p, miR-452-5p, miR-217, miR-196b-5p and miR-9-5p are classified into elevation expression in LIHC (Figure 1), meanwhile, overall survival plot showed that patients with high level expression of miR-9-5p and miR-452-5p respectively following the poorer survival probability(Figure 2), on the another hand, ROC and AUC plots showed the diagnostic and prognostic value of miR-452-5p and miR-9-5p(Figure 3). The expression level of miR-452-5p and miR-9-5p gradually increased with histological grade increased(Figure 4). We introduced the miR-9-5p and miR-452-5p mimic or inhibitor to HepG2 cell line, the results showed that mimic of miR-9-5p or miR-452-5p could promote the cell proliferation and invasion(Figure 5 and 7), while inhibitor of miR-9-5p or miR-452-5p induced the cell apoptosis(Figure 6). These data suggested that miR-9-5p and miR-452-5p could been identified as LIHC prognostic biomarkers.

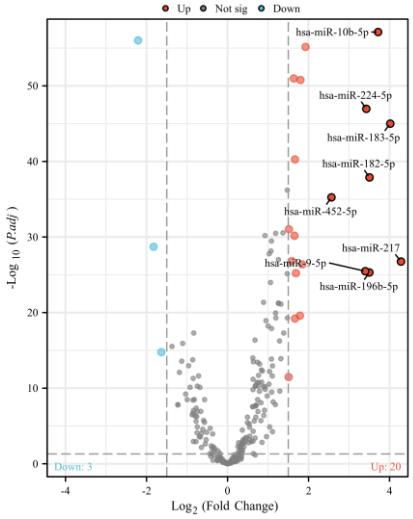

Figure 1 Volcano plot showed the up-regulation and down-regulation miRNAs in LIHC

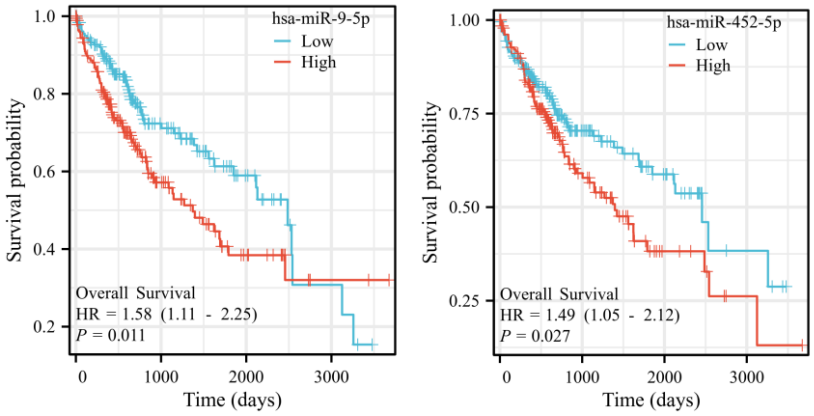

Figure 2 Survival plot, AUC, ROC showed the association between LIHC and miR-9-5p or miR-452-5p

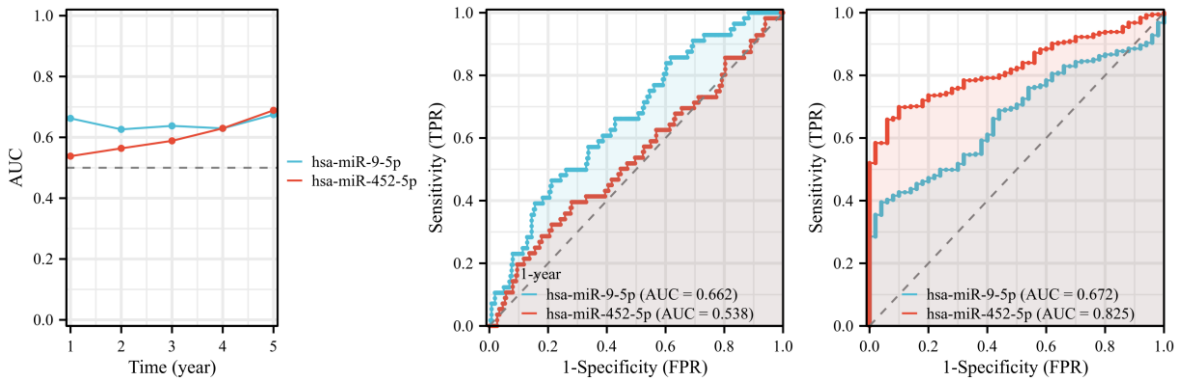

Figure 3 Survival plot, AUC, ROC showed the association between LIHC and miR-9-5p or miR-452-5p

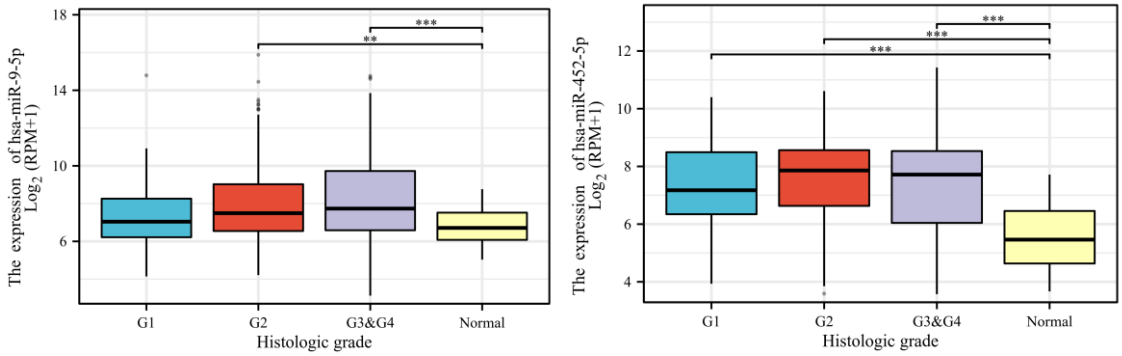

Figure 4 Histologic grade plot demonstrated the association between development of LIHC and miR-9-5p or miR-452-5p

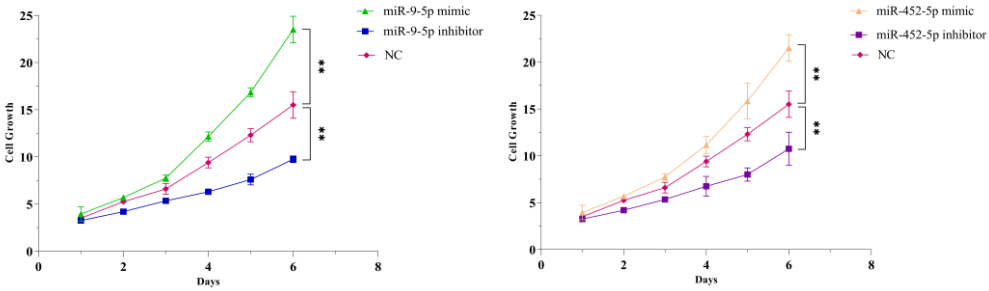

Figure 5 Histologic grade plot demonstrated the association between development of LIHC and miR-9-5p or miR-452-5p

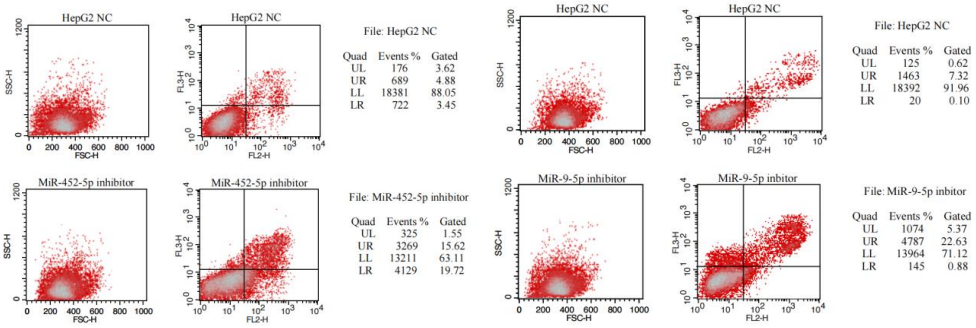

Figure 6 Histologic grade plot demonstrated the association between development of LIHC and miR-9-5p or miR-452-5p

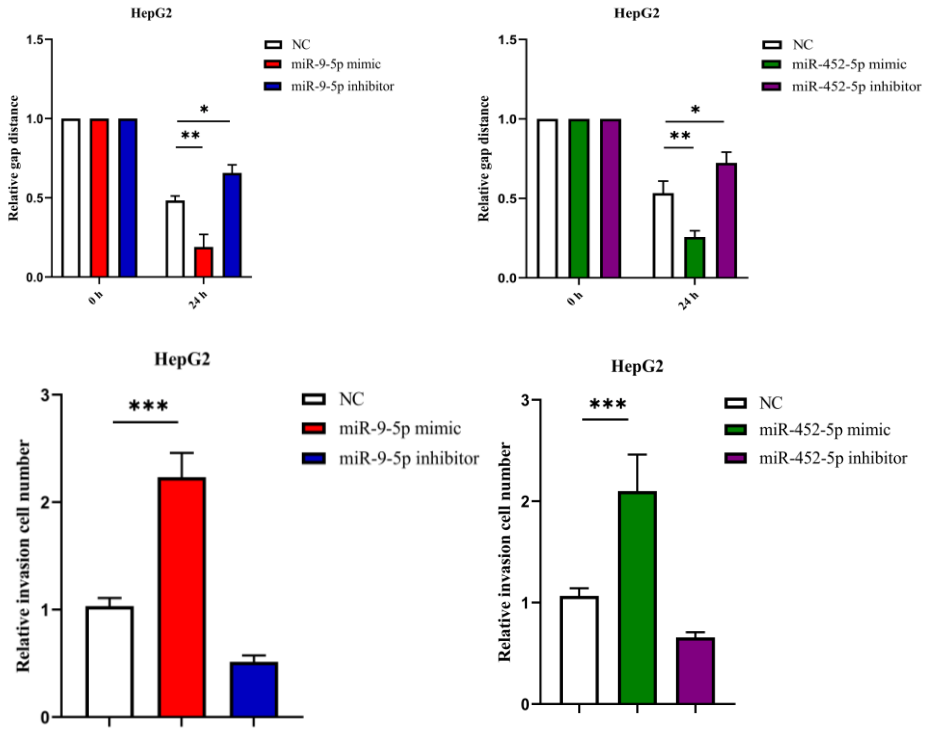

Figure 7 MiR-9-5p and miR-452-5p affect the proliferation and invasion of LIHC

3.2 Identification of miR-9-5p and miR-452-5p targeted proteins in LIHC.

Up-regulation of miRNA in tumor often indicated miRNA specifically suppressed the target proteins, in order to further explore the function of miR-9-5p and miR-452-5p in LIHC, we introduced the TargetScan Human software to generate the targeted proteins, we next introduced the Venn diagram to screen the targeted proteins, group 1 represented the TargetScan Human software generated candidate genes, group 2 represented the down-regulated proteins in LIHC, group 3 represented the prognosis associated proteins.According to results, six proteins in miR-9-5p co-overlapped list, two proteins in miR-452-5p co-overlapped list(Figure 8).We compared the survival plot in co-overlapped list proteins in LIHC, we discovered PDK4 protein and COLEC10 protein(Figure 9).By introduction of TargetScan Human software, we showed the predicted binding sites for PDK4 to miR-9-5p and COLEC10 to miR-452-5p(Figure 10), the luciferase reporter assay showed that miR-9-5p and miR-452-5p could directly bind PDK4 and COLEC10 respectively(Figure 11).Therefore, we found that PDK4 and COLEC10 could been viewed as candidate proteins.

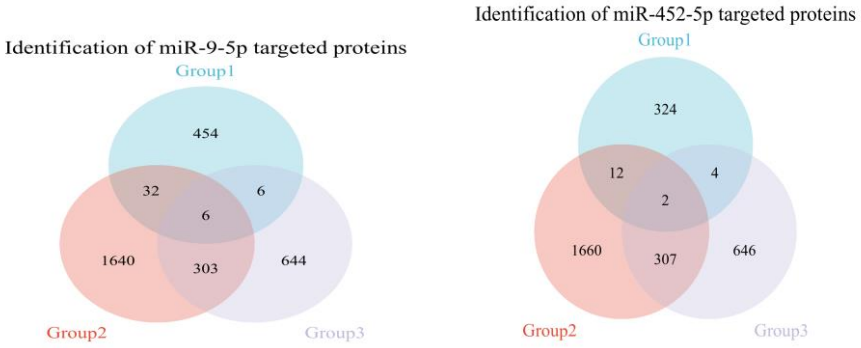

Figure 8 Venn plot showed the overlapping genes of group1 group2 and group3.

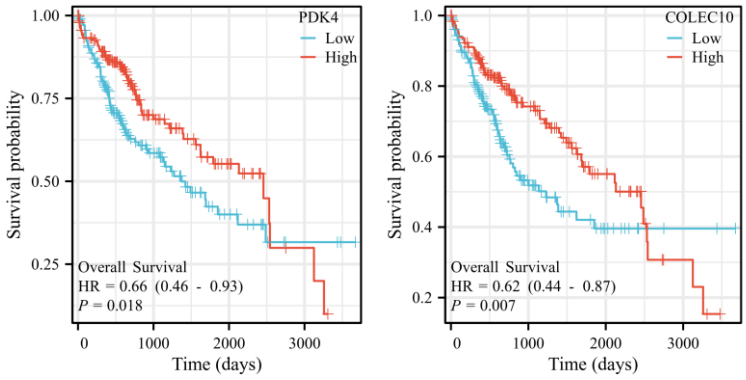

Figure 9 Survival plot of PDK4 and COLEC10 in LIHC

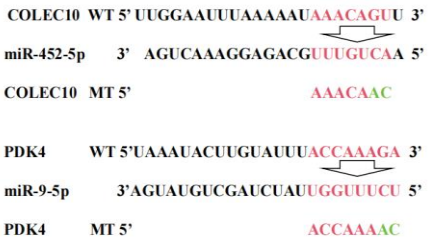

Figure 10 Schematic diagram of the predicted binding sites for miR-9-5p and miR-452-5p on PDK4 3'-UTR and COLEC10 3'-UTR

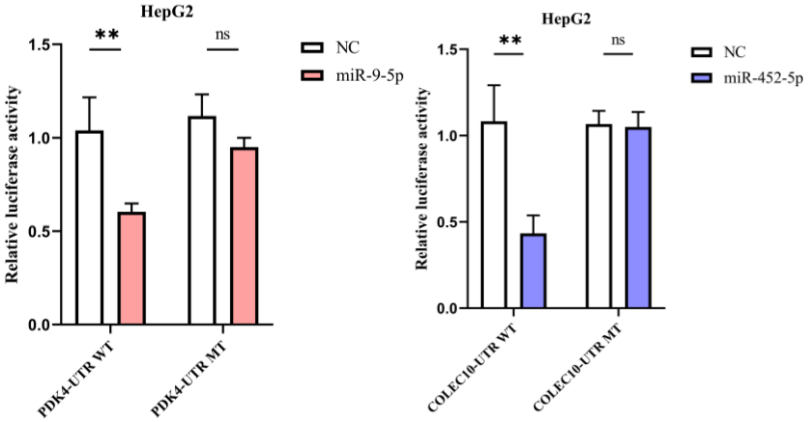

Figure 11 The luciferase activity assays showed the directly binding of miR-9-5p to PDK4 and miR-452-5p to COLEC10

3.3 PDK4 and COLEC10 have clinical significant in LIHC

PDK4(pyruvate dehydrogenase kinase 4), mainly contributed to the regulation of glucose metabolism, has been identified as independent predictor of recurrence in prostate cancer and a key factor to ferroptosis resistance.COLEC10(collectin subfamily member 10), possess collagen-like sequences and carbohydrate recognition domains, specific high-expression in liver. Survival plot showed that LIHC patient with high level of PDK4 and COLEC10 following better survival probability(Figure 9), the violin plot also showed high expression of PDK4 and COLEC10 in adjacent noncancerous liver tissues and low expression in tumor(Figure 12).What's more, the AUC and ROC plots indicate negative association between LIHC development and PDK or

COLEC10(Figure 13).MKI67, marker of proliferation Ki67, is involved in cell division and identified as cancer biomarker, we further explore the relationship between MKI67 and PDK4, COLEC10,the person correlation analysis revealed that PDK4 and COLEC10 were significant negative correlation with MKI67, these two protein mainly enriched in LIHC patients with low MKI67 expression level, while HILPDA, the biomarker of LIHC, is enriched in high MKI67 expression LIHC patients.*ALDH2*, the tumor suppressor gene, is negative link to MKI67 expression(Figure 14).Together, PDK4 and COLEC10 were anti-correlation with LIHC development and progression.

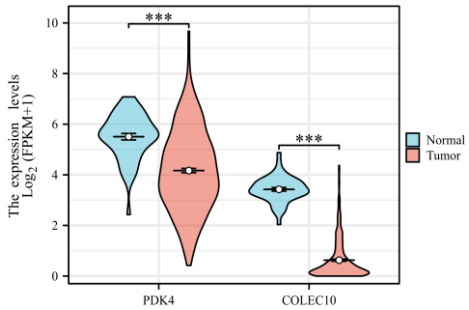

Figure 12 Violin plot showed the distribution of PDK4 and COLEC10 in tumor and normal tissue.

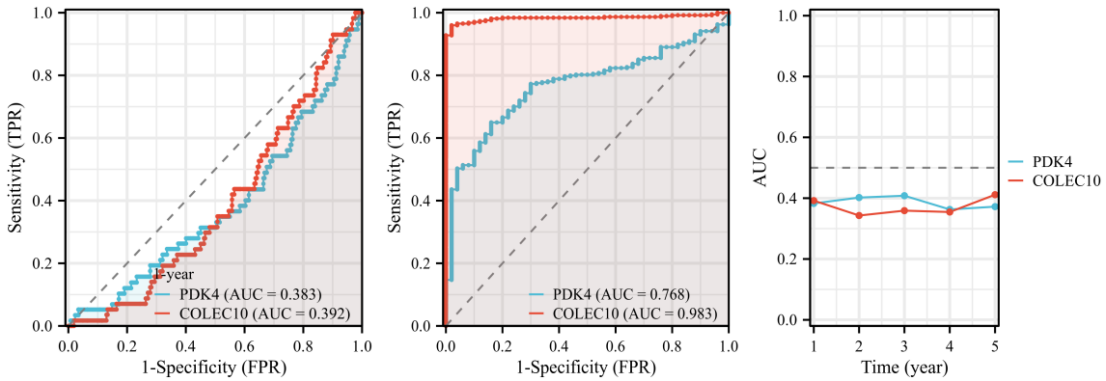

Figure 13 AUC and ROC plot showed the association between LIHC and PDK4 or COLEC10

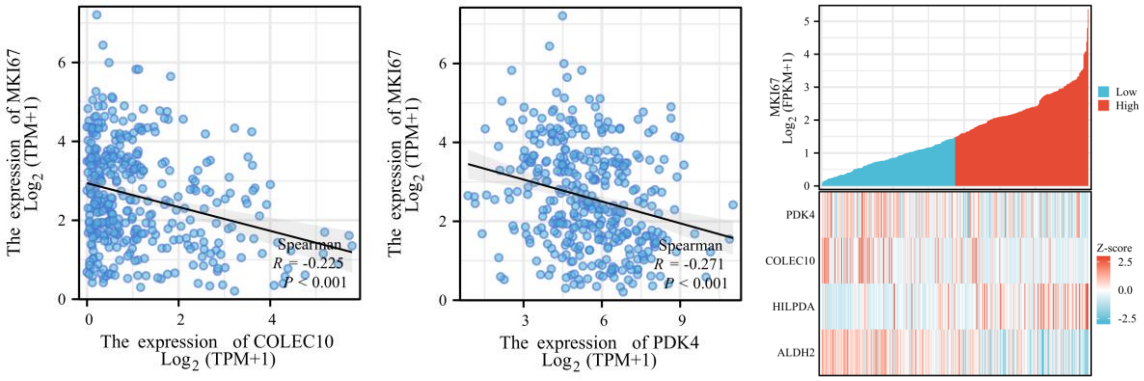

Figure 14 The association between MKI67 and PDK4 or COLEC10

3.4 MiR-9-5p and miR-452-5p regulate LIHC cancer cells through PDK4 and COLEC10

Bioinformatics analysis showed that there was a moderate inverse correlation between LIHC progression and PDK4, COLEC10. We planned to investigate whether elevated expression of PDK4 and COLEC10 could bring effect on LIHC cancer cells. We overexpressed the PDK4 and COLEC10

in HepG2 cell line, the results showed that mimic of miR-9-5p or miR-452-5p could counteract the overexpression of PDK4 or COLEC10, inhibitor of miR-9-5p or miR-452-5p could stimulate the expression of PDK4 or COLEC10(Figure 15). Next, co-transfection of miR-9-5p, miR-452-5p mimics in PDK4 or COLEC10 overexpression cell lines, we could note that PDK4 and COLEC10 could reverse the migration and invasion of miR-9-5p and miR-452-5p(Figure 16 and 17), suggesting the miR-9-5p and miR-452-5p promote the invasion and migration by restriction the miR-9-5p and miR-452-5p.

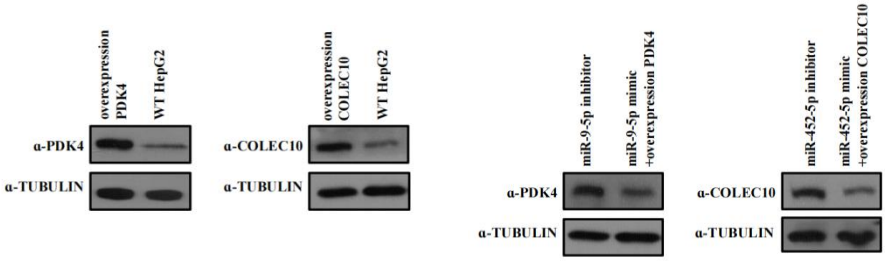

Figure 15 MiR-9-5p and miR-452-5p inhibit the expression of PDK4 and COLEC10

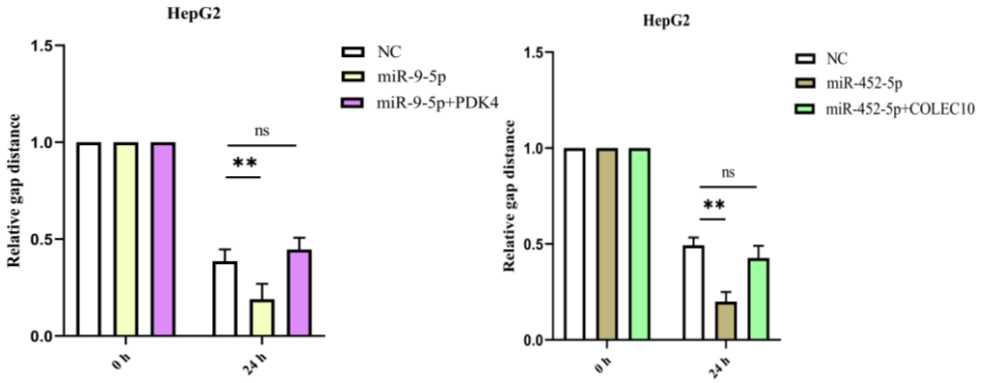

Figure 16 Wound healing and Transwell assays howed that overexpression of PDK4 and COLEC10 reverses the invasive and migrative ability of miR-9-5p and miR-452-5p in LIHC cells.

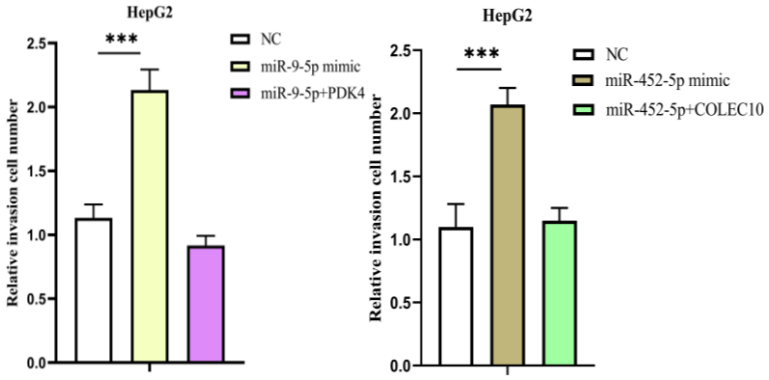

Figure 17 Wound healing and Transwell assays howed that overexpression of PDK4 and COLEC10 reverses the invasive and migrative ability of miR-9-5p and miR-452-5p in LIHC cells.

3.5 Overexpression of PDK4 and COLEC10 significantly alter the malignancy of LIHC

We then performed the colony formation assay and cell derived xenograft assay to test the influence of PDK4 and COLEC10 in cancer proliferation and development, the results showed that overexpression of PDK4 and COLEC10 could dramatically reduce the development and proliferation

of LIHC, indicating the PDK4 and COLEC10 restrict the progression of cancer growth.

3.6 Analysis of genes associated with PDK4 and COLEC10

Based on TCGA database, a total of 84 genes related to PDK4 and 100 genes related to COLEC10 were identified by Person analysis. The correlation between PDK4 mRNA expression and the top 25 of these genes, COLEC10 mRNA expression and the top of these genes were present in(Figure 18), KEGG analysis showed that the enrichment pathways were mainly linked to PPAR signaling pathway in PDK4 related genes and PI3K−Akt signaling pathway in COLEC10 related genes(Figure 19).

Moreover, in order to explore the relevant of PDK4 and COLEC10 in LIHC more comprehensively, the data of PDK4 related genes and COLEC10 related genes were further used to perform GSEA. The GSEA results indicated that the regulation of peroxisome, fatty acid metabolism and epithelian-mesenchymal transition were enhanced in PDK4 and COLEC10 associated genes, whereas, some pathways were inhibited, including G2m checkpoint, MYC targets and E2f targets(Figure 20).

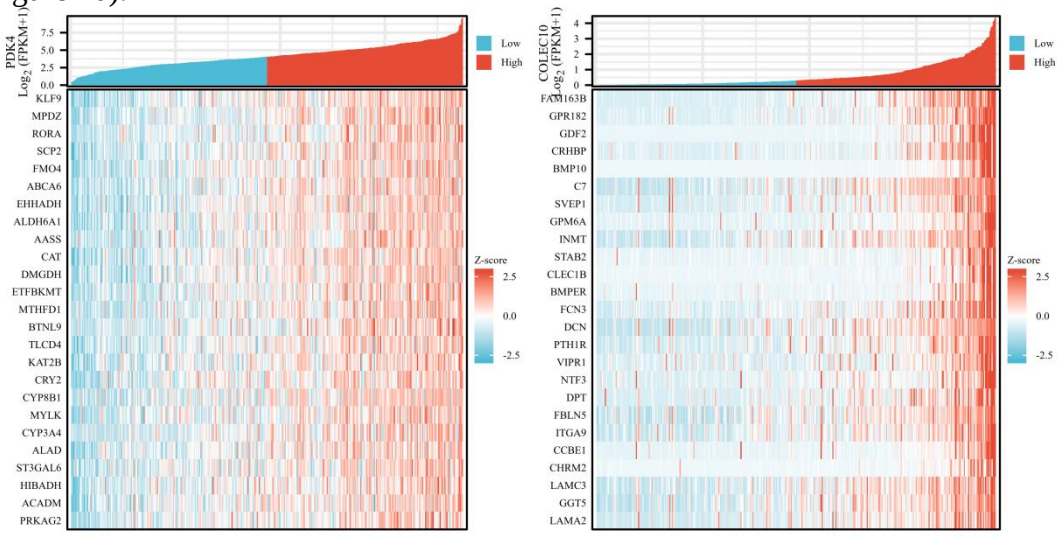

Figure 18 Heat maps identified the PDK4 and COLEC10 associated genes.

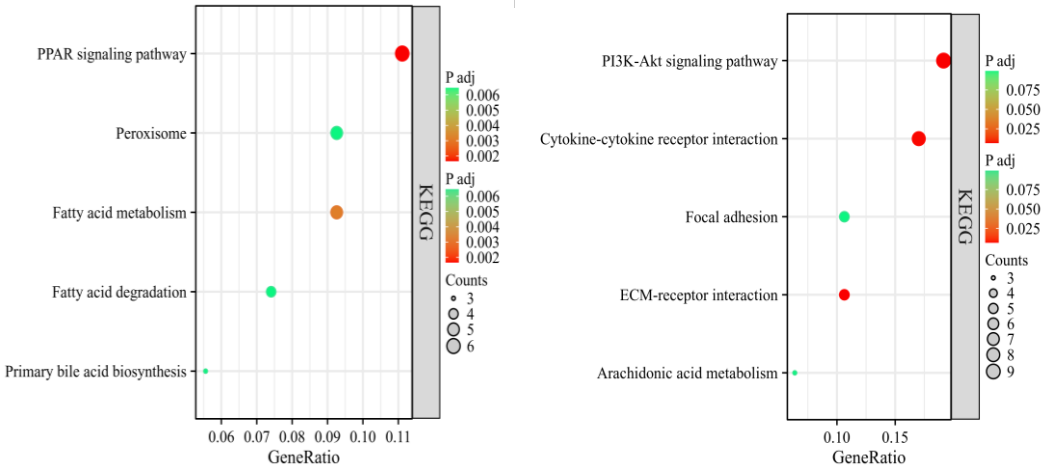

Figure 19 KEGG and GSEA demonstrated the function of PDK4 and COLEC10 associated genes.

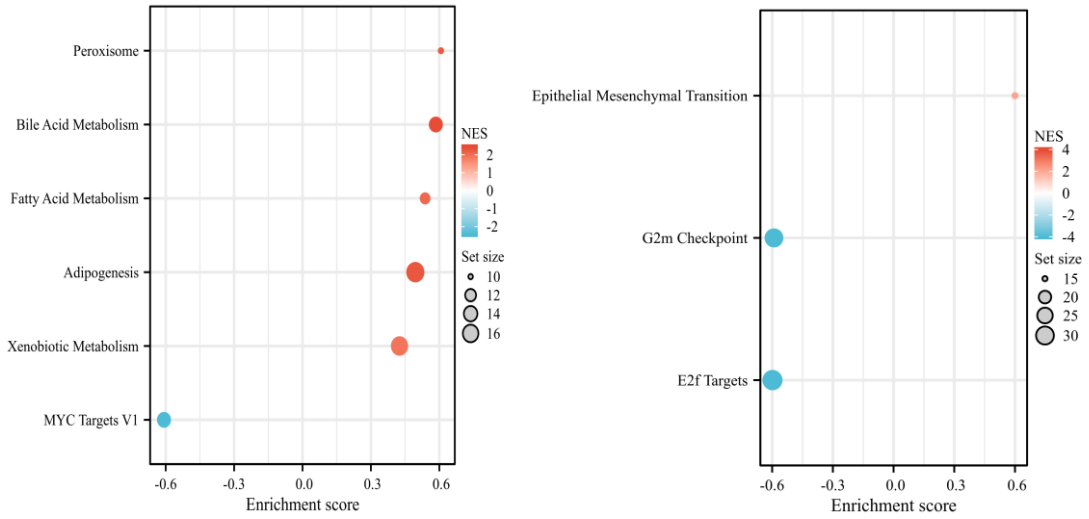

Figure 20 KEGG and GSEA demonstrated the function of PDK4 and COLEC10 associated genes.

4. Discussion

Liver cancer nowadays is a challenging disease around world due to its dismal prognosis and high mortality [3][18], LIHC (hepatocellular carcinoma) is a global life threaten malignancy that has become a global healthcare problem. Despite of great advances in conventional treatment in LIHC, the post-operation survival rate is gloomy, therefore, innovative and low-toxic therapeutic strategy should be further explored [6][8].

miRNA, as a group of short nucleotides in length, is well-known in regulation of genes expression. Recently, its function in tumorigenesis has been shown. Current findings demonstrate that many miRNAs are differentially expressed in LIHC and have essential roles in liver cancer progression through directly targeting a large number of critical genes, indicating the miRNAs could been identified as potential treatment [21][25].

In our researches, we analyzed the 425 specimens data from TCGA, including 50 pan-cancer tissue data and 377 cancer tissue data, by introduction of bioinformatics analysis, we identified up-regulated miRNAs in LIHC cancer, miR-9-5p and miR-452-5p, previous studies shown that miR-9-5p expression is associated with vascular invasion and prognosis, miR-452-5p is up-regulated in various types of cancers but their mechanism in tumorigenesis should be further illustrated [16][26]. In our researches, we also found that patients with high level of miR-9-5p and miR-452-5p have poorer survival rate, then, we identified miR-9-5p targeted protein PDK4, PDK4 acts as oncogene in many cancers by regulation of glucose [24]. We found that PDK4 and its associated genes involved in PPAR signaling pathway and peroxisome activity. COLEC10, belongs to collectin family [20], following low expression in LIHC cancer cells. We uncover that COLEC10 with its related genes mainly disrupted cell cycle by inhibition of G2/M checkpoint by GSEA analysis, KEGG analysis reported these genes involved in PI3K-Art pathway. All in all, the development and progression of cancer is a multiple process [20], networks generated by miRNAs-proteins connections affect cell tumorgenesis [13][25]. In our assays, we bridged the miR-9-5p and PDK4, miR-452-5p and COLEC10 in LIHC cancer cell, KEGG and GSEA analysis noted that activation of PI3K-Art and PPAR signaling pathway may acts as anti-tumor potential therapy.

Conflict of interest

The authors declare that the research was conducted in the absence of any financial relationship

that could be construed as a potential conflict of interest.

Author contributions

Conception: Xiangyu Zhang
Interpretation or analysis of data: Xiangyu Zhang and Yue Li
Preparation of the manuscript: Xiangyu Zhang
Revision for important intellectual content: Xiangyu Zhang
Supervision: Xiangyu Zhang

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