

Distinct Morphological and Molecular Profiles of NAFLD and NAFLD-associated HCC Revealed by Immunohistochemistry and MicroRNA Analysis

Ioana Rusu^{1*}, Radu Pirlog^{2,3*}, Paul Chiroi², Andreea Nutu², Liviuta Budisan², Vlad Radu Puia⁴, Cornelia Braicu², Ioana Berindan-Neagoe², Nadim Al Hajjar^{2,5}

1) Iuliu Hatieganu University of Medicine and Pharmacy, Department of Pathology, Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca, Romania;
2) Iuliu Hatieganu University of Medicine and Pharmacy, Research Center for Functional Genomics, Biomedicine and Translational Medicine, Cluj-Napoca, Romania;
3) Department of Pathology, Institute Curie, Saint-Cloud, France;
4) Iuliu Hatieganu University of Medicine and Pharmacy, 3rd Department of General Surgery, Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca, Romania

Address for correspondence:

Ioana Berindan-Neagoe
Iuliu Hatieganu University of Medicine and Pharmacy, Research Center for Functional Genomics, Biomedicine and Translational Medicine, Cluj-Napoca, Romania
ioananeagoe29@gmail.com

Received: 08.06.2023

Accepted: 28.08.2023

*: equal contribution

ABSTRACT

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is a common hepatic condition that can progress to hepatocellular carcinoma (HCC) in non-cirrhotic livers. To better understand the development of NAFLD-associated HCC, we performed an integrated morphological and molecular analysis to identify new insights that can improve the follow-up of NAFLD patients.

Methods: Our study included a cohort of 14 NAFLD-associated HCC and 41 NAFLD patients. We analyzed clinical parameters, a four-microRNA (miRNA) panel (miR-21-5p, miR-34a-5p, miR-130a-3p, and miR-155-3p) panel and their relationship with p53 and β -catenin expression.

Results: In the study cohort, the NAFLD-associated HCC patients were predominantly male, older, had significantly altered hepatic function, and a higher incidence of hypertension, type 2 diabetes, and dyslipidemia. Morphologically, the NAFLD-HCC group had substantially higher steatosis, ballooning, and fibrosis grades than the NAFLD group. The β -catenin expression was higher in both adjacent non-tumoral liver tissue (ANT) from NAFLD-associated HCC patients and in HCC tissue compared with NAFLD samples. The 4 miRNAs panel showed a dysregulated expression profile between NAFLD, ANT and HCC samples.

Conclusions: This study provides important insights regarding the molecular mechanisms underlying HCC progression in NAFLD patients, allowing for the development of better screening strategies for the early detection of NAFLD-associated HCC.

Key words: non-alcoholic fatty liver disease – NAFLD - hepatocellular carcinoma – HCC – microRNAs – miRNAs – cancer progression – biomarkers.

Abbreviations: ALP: alkaline phosphatase; ALT: alanine aminotransferase; ANT: adjacent non-tumoral liver tissue; AST: aspartate aminotransferase; EMT: epithelial-mesenchymal transition; FFPE: formalin-fixed paraffin-embedded; GGT: gamma-glutamyl transferase; HCC: hepatocellular carcinoma; IHC: immunohistochemistry; INR: international normalized ratio; microRNA: miRNA; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; ncRNA: non-coding RNA; NLR: neutrophil-to-lymphocyte ratio; T2D: type 2 diabetes; TBIL: total bilirubin.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a frequent cause of chronic liver disease. It affects 24% of the global population and is frequently associated with obesity and type 2 diabetes (T2D) [1-3]. The histological spectrum of NAFLD varies from simple steatosis, or non-alcoholic fatty liver (NAFL), to non-alcoholic steatohepatitis (NASH), characterized by

lobular inflammation and hepatocellular ballooning, with or without fibrosis. Non-alcoholic fatty liver disease is the hepatic manifestation of different metabolic disorders; 50% of the individuals with T2D and 90% of the morbidly obese have NAFLD. This hepatic condition is the most prevalent cause of chronic liver disease, affects over 25% of the general population in industrialized nations, and is also the leading cause of HCC development in non-cirrhotic liver disease [4].

The global incidence of HCC increased by 75%, becoming the second leading cause of years of cancer loss [4, 5]. Almost 50% of patients with NAFLD-associated HCC had no evidence of cirrhosis [4, 6]. Although viral hepatitis remains the primary cause for about 60-85% of the worldwide HCC cases, it is estimated that NAFLD accounts for 30-40% of these scenarios

[7, 8]. Non-alcoholic fatty liver disease associated HCC cases are usually clinically silent during the early stages [9, 10] and has a less aggressive phenotype, so it can be easily missed on routine scans [11, 12].

A growing body of evidence confirms the regulatory role of small non-coding RNAs (ncRNAs) on the expression of TP53 and the synthesis of β -catenin during HCC pathogenesis. Consequently, novel HCC biomarkers were discovered, including a vast repertoire of ncRNAs [13-16]. Dysregulation of these short non-coding transcripts was associated with several hallmarks of HCC, such as proliferation, apoptosis, invasion, metastasis, epithelial-mesenchymal transition (EMT), angiogenesis, drug resistance and autophagy, so they hold great promise for enabling a better overview of the molecular mechanisms underlying HCC progression [17, 18]. During the last decade, a growing body of evidence started to back up the role of miRNAs as pivotal players in the non-cirrhotic NAFLD-HCC [19-21].

While most published articles focus on HCC alone, more data is needed to evaluate the miRNA profile dynamics between the healthy liver, NAFLD and NAFLD-associated HCC [22, 23]. Based on this literature gap we identified a panel of four miRNAs, miR-21-5p, miR-34a-5p, miR-130a-3p and miR-155-3p and analyzed their expression and association with NAFLD progression towards HCC.

METHODS

Patient Information

A retrospective cohort of 14 NAFLD associated-HCCs and 41 NAFLD formalin-fixed paraffin-embedded (FFPE) samples from Prof. Dr Octavian Fodor Regional Institute of Gastroenterology-Hepatology, Cluj-Napoca, Romania, diagnosed between January 2012 and March 2021, was included in the present study. This study was approved by the Ethics Committees of the Prof. Dr Octavian Fodor Regional Institute of Gastroenterology-Hepatology, Cluj-Napoca, Romania (1241/27.01.2021) and the Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania (382/23.12.2020). Patients with alcoholic liver injury, autoimmune hepatitis, and other liver disease superimposed to NAFLD and cirrhosis were excluded. All NAFLD patients included in the study were HBV- and HCV-negative. All patients included in the study signed the informed consent.

Data Collection

Clinical and laboratory data were obtained through a retrospective review of patients' files. Baseline clinical data were collected at the index date, including sex, age, medical history (T2D, obesity, dyslipidemia, and hypertension) and blood parameters: international normalized ratio (INR), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP) and neutrophil-to-lymphocyte ratio (NLR).

Morphological Characterization

Histological examination of the specimen was done on hematoxylin-eosin slides aided by special stain trichrome

Masson. Sections from each specimen were examined by two pathologists (I.R and R.P.). For all NAFLD specimens, the Brunt scoring system was used to determine the grade of inflammation, steatosis, ballooning, and stage of fibrosis [24]. For NAFLD-associated HCC cases, the staging was done according to the WHO Classification of Tumors 2019. Parameters analyzed for HCC cases were grading, stage and presence of lymphovascular invasion.

Immunohistochemistry

Immunohistochemistry (IHC) staining was performed using a fully automated slide preparation system (Leica automatic Bond-Max system). IHC staining was performed for the proteins of β -catenin (Abcam, 17C2 clone) and p53 (Abcam, DO-7 clone). β -catenin staining (nuclear and/or cytoplasmic) was scored according to intensity (0–no staining, 1–weak, 2–moderate, and 3–strong), both in NAFLD tissue and HCC tissue. Nuclear p53 expression was interpreted as negative when heterogenous staining was seen (score 0) and as positive when a diffuse overexpression was seen (score 1) [25].

Bioinformatics Analysis

To better understand the regulatory network of the four miRNAs panel (miR-21-5p, miR-34a-5p, miR-130a-3p, and miR-155-3p) and HCC, we used miRNET to generate and analyze miRNA-gene interaction (<https://www.mirnet.ca/miRNet/faces/home.xhtml>). Additionally, we developed a heatmap for the 4-miRNA panel to assess their expression across various cancers and metabolic processes [26]. The expression of 4 miRNAs in HCC vs normal tissue and their impact on patient survival was bioinformatically assessed using the data from The Cancer Genome Atlas (TCGA) and the Starbase online tool [27].

RNA Extraction

RNA was extracted from FFPE tissue using the automated Maxwell® RSC RNA FFPE Kit (Cat. No. AS1440) based on the instructions furnished by the manufacturer's protocol. To better understand the progressive changes in miRNA expression, we extracted RNA from three different sample types: 41 NAFLD samples, 14 adjacent non-tumoral tissue from NAFLD-associated HCC patients (ANT) and 14 tumoral samples from NAFLD-associated HCC patients. The total RNA extracted from the FFPE Tissue Sections was quantitatively and qualitatively measured with a NanoDrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

cDNA and qRT-PCR

The cDNA synthesis was done using 100 ng of total RNA extracted from FFPE tissue. Based on the recommended protocol, the cDNA synthesis was realised using 100 ng of total RNA extracted from FFPE tissue, using the miScript HiSpec Buffer in a 20 μ L and the following program: 37°C for 60 minutes and then at 95°C for 5 minutes. The cDNA was then diluted for a PCR array based on a SybrGreen protocol. The miRNA expression level was evaluated using a TaqMan Based protocol and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). For the amplification, we used TaqMan Fast Advanced Master Mix (Applied

Table I. Mature sequences of the normalizers and miRNA that were used in the study

miRNA	Mature sequence
RNU48	GATGACCCCAGGTAACCTCTGAGTGTGTGCTGATGCCATCACCGCAGCGCTCTGACC
U6	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAG GAAGATTAGCATGGCCCTGCGCAAGGATGACACGCAAATTCGTG AAGCGTCCATATTTT
miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
miR-155-3p	CUCCUACAUAUUAGCAUUAACA
miR-34a-5p	UGGCAGUGUCUUAGCUGGUUGU
miR-130a-3p	CAGUGCAAUGUAAAAGGGCAU

Biosystems) and TaqMan™ MicroRNA Assay: RNU48 (Cat. No. 4427975); U6 (Cat. No. 4427975); miR-21-5p (Cat. No. 4427975); miR-155-3p (Cat. No. 4427975); miR-34a-3p (Cat. No. 4427975); miR-130a-3p (Cat. No. 4427975) on the same instrument. Mature sequences of the miRNA and normalizers are presented in Table I.

Statistical Analysis

The qRT-PCR data analysis was achieved using the $\Delta\Delta C_t$ method, as previously described by Berindan-Neagoe et al. [28]. Statistical data analysis was performed with Prism software version 8.0. The t-test was used to compare the differential expressions of miRNAs between tumor tissue, NAFLD tissue and adjacent normal tissue. To compare differences between the morphological and immunohistochemical characteristics, the chi-squared test and Fisher's exact test were used. A p-value lower than 0.05 was considered statistically significant.

RESULTS

Patients' Cohort Clinical Characteristics

From January 2012 to March 2021, 14 patients with HCC developed in a background of non-cirrhotic NAFLD who underwent hepatectomy, and 41 specimens from patients with NAFLD from non-HCC patients, biopsy-proven, were included in our cohort. The baseline characteristics, sex distribution and associated comorbidities of the patient groups, and an analysis of the associated differences are summarized in Table II.

There was a difference in gender distribution in our study cohort, as the patients with NAFLD who progressed towards HCC were all male. The mean age difference between the two groups was statistically significant with a positive 17-year difference (50.7 vs 68.7) for the NAFLD-associated HCC group. Hepatic function was significantly altered in the NAFLD-associated HCC groups with significant differences compared with NAFLD patients for the levels of ALT ($p=0,042$) and bilirubin ($p=0,027$).

The existing comorbidities in the two groups varied significantly, with HTA being present in 78.5% of NAFLD-HCC (11/14) compared with 41.4% in the NAFLD group (17/41) ($p=0.028$). T2D and dyslipidemia were present in all 14 patients with NAFLD-HCC. NAFLD patients presented T2D in 39% of cases (16/41) and dyslipidemia in 56% (23/41). There were no significant differences regarding obesity between the two groups.

Table II. Baseline characteristics and clinical parameters of the patient cohort

Parameter	NAFLD	NAFLD-related HCC	p
Gender			
M	20	14	0.0004
F	21	0	
Mean age, years, range	50.7 (23-67)	68.7 (57-77)	<0.0001
INR	1.05 (0.84-1.6)	1.06 (0.98-1.23)	0.8262
AST (UI/ml)	73,92 (12-444)	30.35 (15-56)	0.0614
ALT (UI/ml)	111 (10-1288)	31.35 (9-75)	0.0427
TBIL (UI/ml)	1,47 (0,3-13,9)	0.81 (0.36-2.1)	0.0270
GGT (UI/ml)	170,09 (22-796)	62.57 (33-163)	0.0763
ALF (UI/ml)	265.63 (26-734)	252.07 (137-511)	0.7013
NLR	2.32 (0,72-4,63)	2.91 (0,87-6,96)	0.0983
AST (UI/ml)	73.92 (12-444)	30.35 (15-56)	0.0614
HTA			
Yes	17	11	0.0286
No	24	3	
T2D			
Yes	16	14	<0.0001
No	25	0	
Dyslipidemia			
Yes	23	14	0.0021
No	18	0	
Obesity			
Yes	15	3	0.3457
No	26	11	

INR: international normalized ratio; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBIL: total bilirubin; GGT: gamma-glutamyl transferase; ALF: alkaline phosphatase; NLR: neutrophil-to-lymphocyte ratio; HTA: hypertension; T2D: type 2 diabetes.

Morphologic Characteristics

HCC samples were classified based on their grade of differentiation in three groups. A graphic representation of the three differentiation stages is presented in Fig. 1.

Data regarding the grade of steatosis, balonisation, fibrosis and abundance of associated inflammation present in the tissue of NAFLD and NAFLD-HCC patients are summarized in Table III.

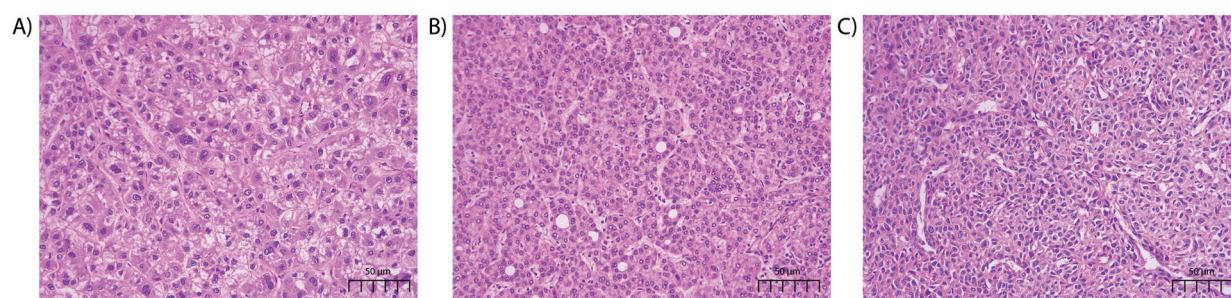


Fig. 1. Hepatocellular carcinoma degree of differentiation in NAFLD progressed HCC. A) Grade III poorly differentiated HCC with solid masses of cancer cells, disorganized architecture, and high cellular polymorphism, HE 200x magnification; B) Grade I well-differentiated HCC with the majority of cells resembling adjacent liver tissue, HE 200x magnification; C) Grade II moderately differentiated HCC with tumor cells still having the liver-like organization, with zones of solid architecture, HE 200x magnification.

Table III. Morphological characterization of the histological parameters between the two groups

Parameter	NAFLD	NAFLD-related HCC	p
Steatosis			0.0012
1	8	10	
2	15	3	
3	18	1	
Baloonisation			<0.0001
0	1	8	
1	30	6	
2	10	0	
Fibrosis			0.0124
1	16	0	
2	18	8	
3	7	8	
Inflammation			0.7015
1	31	7	
2	10	3	

The two groups showed similar levels of inflammation in the liver tissue, with higher levels seen in the NAFLD patient cohort (Fig. 2). Steatosis was significantly more severe in the NAFLD group, with 33 patients in grade 2 or 3, NAFLD-HCC patients showed a lower level of steatosis with 10/14 showing a grade 1 steatosis (Fig. 3) ($p=0.001$).

Hepatocyte baloonisation was significantly more pronounced in the NAFLD group with 40 (97%) of patients showing a grade 1 or grade 2 ($p<0.0001$) and only 6 (42.8%) patients from the NAFLD-associated HCC showing a grade 1 baloonisation of hepatocytes ($p<0.0001$). Fibrosis was significantly more pronounced in NAFLD-HCC samples. All patients showed at least a grade 2 level of fibrosis with 6 (42.8%) showing a grade 3 level of fibrosis; in the NAFLD group 34 (82.9%) showed a low to moderate fibrosis level ($p=0.012$).

Immunohistochemistry Characterization

β -catenin and p53 IHC expression were assessed in the two patient cohorts (Table IV). β -catenin showed low expression (score 0 and 1) in 39 (95.1%) of NAFLD samples. Higher expression was seen in both ANT and NAFLD-associated HCC samples, with 11 (78.6%) (cases in both ANT and NAFLD-

Table IV. β -catenin and p53 IHC analysis in NAFLD and NAFLD-associated HCC samples

IHC Marker	NAFLD	ANT	NAFLD-associated HCC	p
β -catenin				<0.0001
0	34	0	0	
1	5	2	3	
2	1	7	9	
3	1	4	2	
p53				<0.0001
0	41	14	5	
1	0	0	9	

associated HCC samples) showing a moderate to intense score of β -catenin, the difference being statistically significant between the two cohorts ($p<0.0001$) (Fig. 4, A-G). p53 IHC expression assessment showed significant profile changes between the cohorts with p53 being negative, non-mutated in all NAFLD and ANT samples and showing a positive expression in 9 (64.3%) of NAFLD-associated HCC cases (Fig. 5).

Bioinformatics Analysis of the Four-miRNA Panel

A heatmap was generated using the Starbase V.8 online software to analyze the expression level of the 4-miRNA panel in cancer and cancer-related metabolic processes (Fig. 6). Three miRNAs from our panel (miR-21-5p, miR-130a-3p and miR-34a-5p) are overexpressed in p53 signaling pathway, in proteoglycans processing pathway in cancer and in hepatitis B. Three miRNA, miR-21-5p, miR-130a-3p and miR-34a-5p are involved in fatty acids metabolism and cell cycle progression. Additionally, miR-34a-5p and miR-130a-3p are overexpressed in multiple solid malignancies and in oncogenic pathways.

Expression Level Analysis of the Four-miRNA Panel

The expression of the 4-miRNA panel was assessed on the TCGA database HCC samples. miR-21-5p and miR-34a-5p were overexpressed in HCC tissue samples compared with normal control samples ($p<0.0001$). MiR-130a-3p was downregulated in HCC samples compared with normal controls ($p<0.0001$) and for miR-155-3p no statistically significant differences between HCC samples and controls were seen ($p=0.86$) (Fig. 7).

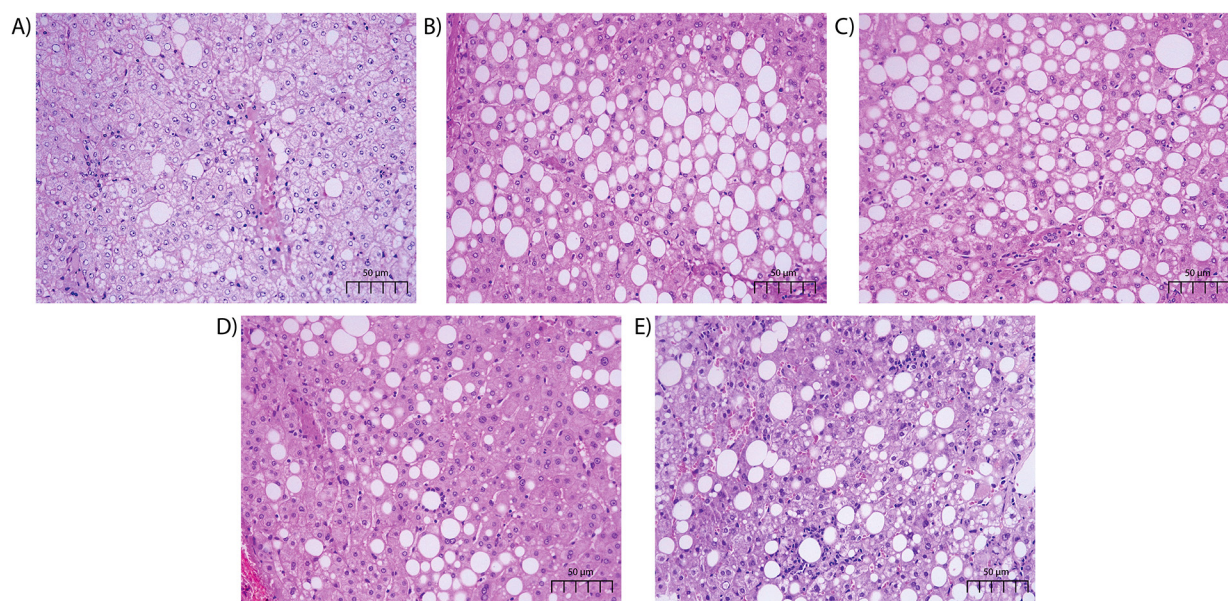


Fig. 2. Morphological assessment of the study samples' degree of steatosis and inflammation. A) Steatosis grade 1: rare lipid inclusions of small and medium size, HE 200x magnification. B) Steatosis grade 2 with a more pronounced hepatocytes accumulation, HE 200x magnification. C) Steatosis grade 3 with multiple intracytoplasmic lipid inclusions affecting normal liver architecture. D) NAFLD liver tissue with several lymphocytes, inflammation score 1, HE 200x magnification. E) NAFLD with inflammation score 2.

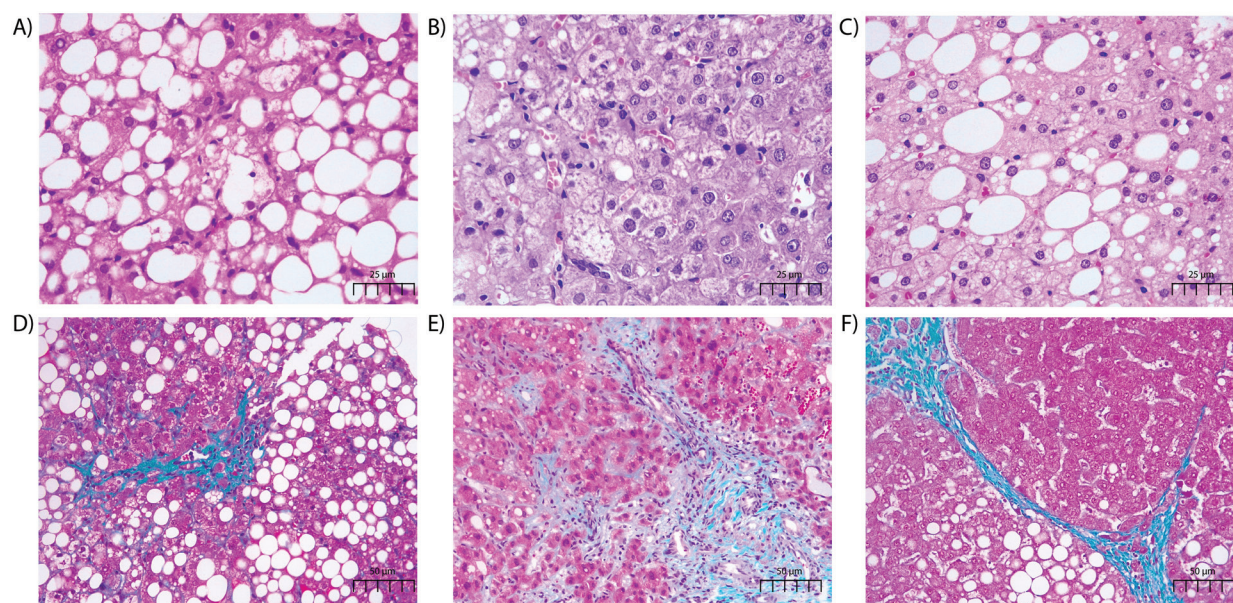


Fig. 3. Evaluation of the ballooning and fibrosis grade in NAFLD and NAFLD-HCC samples on HE and Trichrome staining. A) Occasional ballooned hepatocytes (star) observed (grade 1) HE 200x; B) Many ballooned hepatocytes observed (grade 2) HE 200x; C) No ballooned hepatocytes observed (grade 0) HE 200x; D) Perisinusoidal fibrosis observed in zone 3 (stage 1)-Masson trichrome stain. E) Perisinusoidal and portal/periportal fibrosis, without evidence of bridging fibrosis (stage 2) Masson trichrome stain; F) Occasional septal fibrosis without evidence of cirrhosis (stage 3) Masson trichrome stain.

Expression Level of the Selected MiRNA Panel Using qRT-PCR Expression Analysis

We aimed to analyze the miRNAs expression dynamics during NAFLD progression towards HCC. The differential expression analysis revealed statistically significant up-regulation of miR-21-5p and miR-34a-5p in peritumoral tissues (ANT) compared with an even higher statistically significant up-regulation in tumor tissues when compared with ANT and NAFLD samples. This expression profile highlights a steady progression in the expression levels of these two miRNAs from

NAFLD to ANT and NAFLD-associated HCC (Fig. 8 A and D). The miR-155-3p expression was also statistically significant up-regulated in the ANT and HCC samples compared to the NAFLD samples (Fig. 8 B). Still, there was no statistically significant difference in its expression level when miR-155-3p was compared between the ANT and HCC samples. The expression of miR-130a-3p was significantly up regulated in the ANT samples compared to NAFLD and HCC tissues (Fig. 8 C). There was no statistically significant difference in miR-130a-3p expression compared to NAFLD and HCC samples (Fig. 8 C).

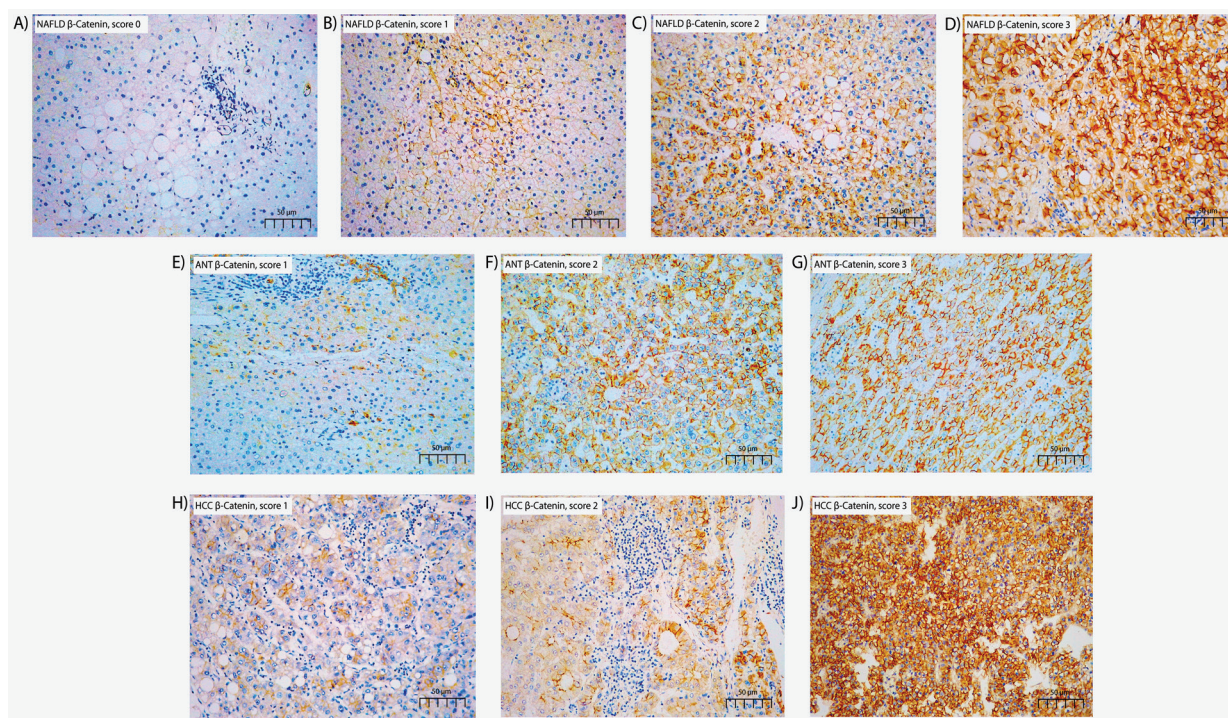


Fig. 4. β -catenin IHC expression quantified on a three-level scale from 1 (low) to 3 (intense). In both NAFLD, ANT and HCC tissue. A-D) NAFLD samples, β -catenin staining, 200x. E-G) ANT samples, β -catenin staining, 200x. H-J) HCC samples, β -catenin staining, 200x.

DISCUSSION

This study investigates the changes in the expression profile of a four-miRNAs panel in NAFLD and NAFLD-associated HCC, focusing on clinical and genomic alterations to better understand the HCC tumorigenesis on the frames of a pre-existing NAFLD. A significant difference was seen between the mean age at diagnosis between the two groups, with an 18-year

difference in favor of the NAFLD-associated HCC, suggesting that the progression from NAFLD to HCC is a long process that could be prone to screening if adequate strategies are being implemented. Due to the relatively low incidence of malignant transformation and lack of clear guidelines, regular screenings by liver fibrosis assessment are not recommended in low-risk patients. Also, there need to be clear follow-up guidelines for medium and high-risk patients [29]. Thus, this surveillance

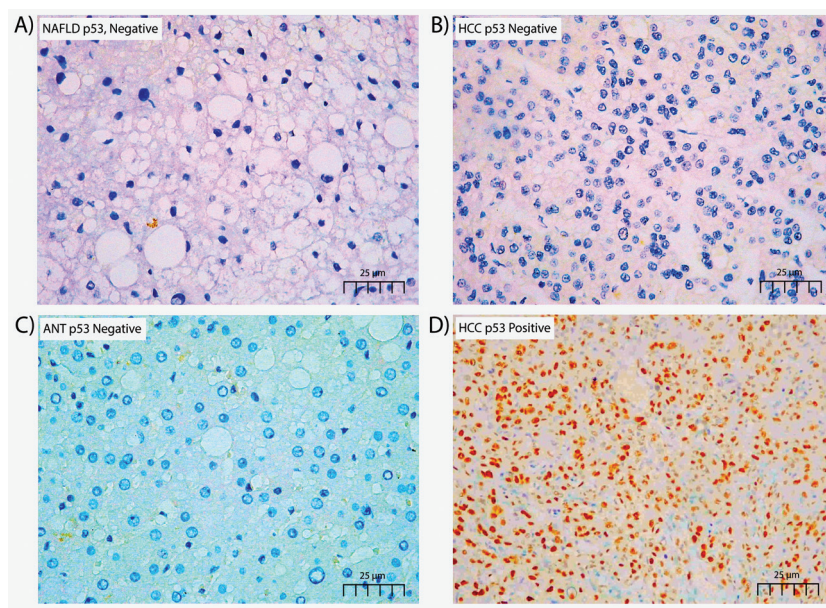


Fig. 5. p53 IHC staining quantified on a dichotomic scale of negative and positive. A, B, C) p53 negative IHC staining in NAFLD, ANT and HCC samples, 200x. D) p53 positive staining in HCC samples, 200x.

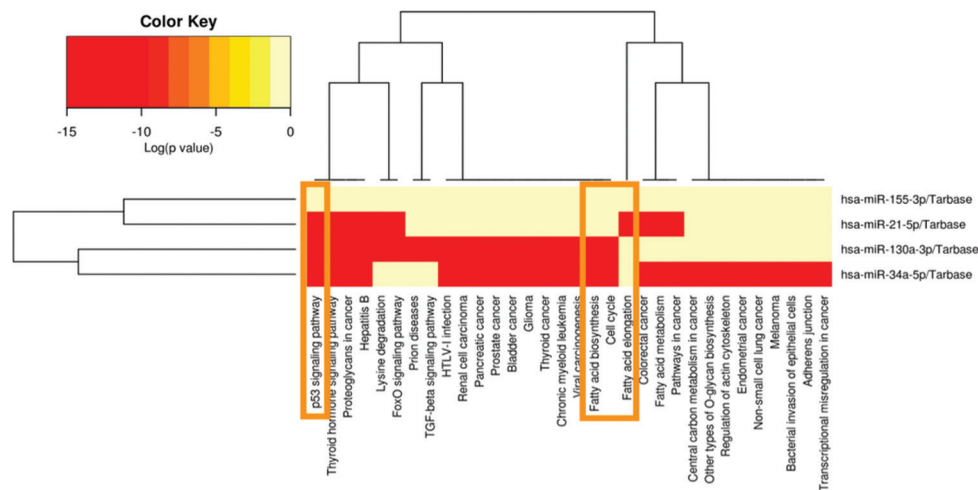


Fig. 6. Heatmap analysis of the expression levels of the 4-miRNA panel in different cancers and cancer-associated metabolic processes according to the Starbase database. The two orange squares are highlighting the important pathways related to NAFLD in which the 4 miRNAs are involved: p53 signaling pathway and fatty acid related pathways.

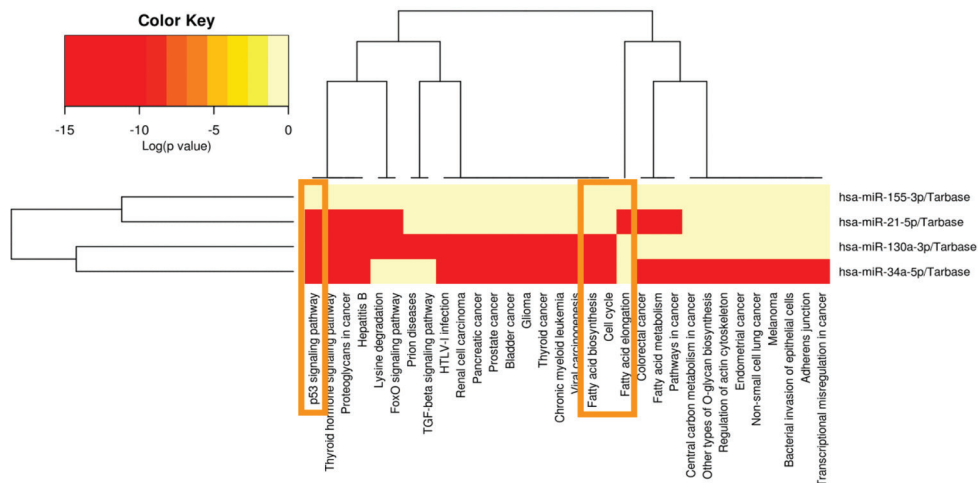


Fig. 7. Expression level analysis, the four-miRNA panel in HCC tissue compared to normal liver tissue, according to the TCGA data analysis. ns – nonsignificant; **** p<0,0001.

gap raises the requirement for more accurate, non-invasive, and low-cost screening methods to identify NAFLD's early progression towards HCC.

In our study, parameters assessing liver function were significantly different between the two cohorts, with a lower level of AST, ALT, TBIL, GGT, and ALP in NAFLD-associated HCC patients, which was expected given the advanced stages of fibrosis in this cohort. Higher NLR levels were observed in patients with NAFLD-HCC related. The NLR, frequently used to assess inflammatory disorders, measures the absolute quantity of neutrophils in lymphocytes [30]. Our findings are in concordance with the evidence from a recent study by Thomas et al. [31], who showed that patients with NAFLD with higher NLR counts have a greater chance of developing HCC.

We observed a statistically significant difference for HTA, T2D and dyslipidemia between the two cohorts, with these comorbidities being more prevalent in the NAFLD-associated HCC group. Morphologic assessment in hematoxylin-eosin

staining showed differences in steatosis and hepatocyte balonisation grade. A lower grade of steatosis and balonisation in NAFLD-associated HCC is possible due to the affected liver function in the context of HCC. The level of fibrosis was more elevated in NAFLD-associated HCC, with all patients having at least a grade II level of fibrosis and 42% of them in the precirrhotic stage. Interestingly there were no significant differences regarding the level of associated inflammation between the two cohorts, suggesting that the tumorigenesis mechanisms in these patients are caused mostly by metabolic alterations and is not related to the inflammatory pathways as is seen in NASH or viral-associated HCC [32-34].

We witnessed significant differences in β -catenin expression between the study cohorts (NAFLD, ANT and NAFLD-associated HCC). As such, the overexpression of β -catenin in ANT and NAFLD-associated HCC cases suggests that one of the mechanisms involved in the progression of NAFLD towards HCC is the dysregulation of the WNT

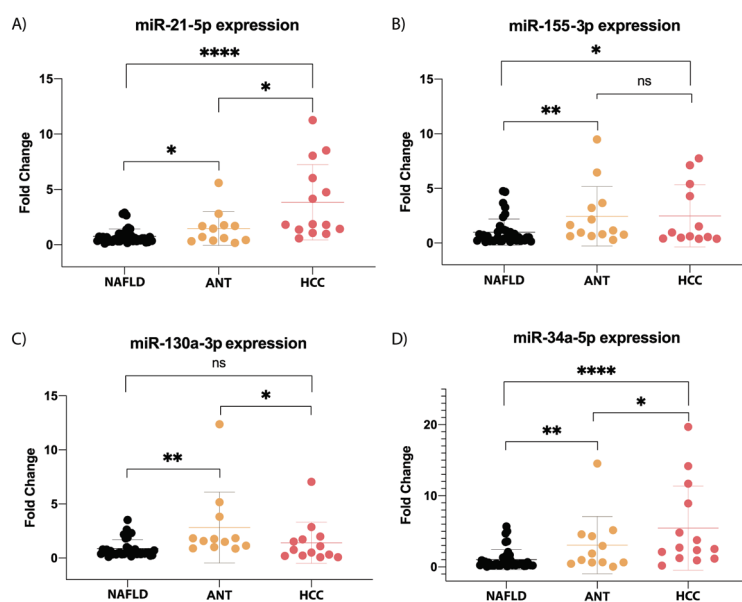


Fig. 8. Differential expression analysis using qRT-PCR of the four-miRNA panel in NAFLD and NAFLD associated-HCC samples. NAFLD tissue; ANT: adjacent NAFLD non-tumoral tissue from NAFLD-associated HCC patients; HCC: tumor tissue from NAFLD - associated HCC patients.

signaling pathway. Kim et al. [35] showed that β -catenin is a major oncogene in HCC development, with WNT pathway dysregulation being an important early genetic event during tumorigenesis. β -catenin mutations and alterations increase cytoplasmic levels and nuclear accumulation, as shown by the IHC staining. In addition, Wong et al. [36] demonstrated a robust „field effect” in the livers of individuals with NAFLD and HCC, where activated β -catenin changed the immune microenvironment. This “field effect” in NAFLD patients can be easily highlighted with IHC staining. Interestingly, β -catenin was elevated in ANT tissue at significantly higher levels than in NAFLD cases, with only 2 cases out of 41 (4.8%) in the NAFLD group showing a moderate or high expression. This result suggests that β -catenin may provide important insights of a possible cancerization effect in NAFLD that could identify patients at risk of further HCC development.

P53 IHC was evaluated to assess the presence of TP53 gene alterations, as it can be an accessible surrogate marker [37, 38]. Immunohistochemistry staining detected p53 alterations only in NAFLD-associated HCC samples (62%). TP53 gene is an important tumor suppressor frequently mutated in HCC, its mutation allowing hepatic cells to escape the cell cycle surveillance and promote uncontrolled division and tumorigenesis [39]. TP53 gene activity can be indirectly dysregulated by miRNA modulations, as it can downregulate its expression, thus limiting its function [40, 41]. Also, miR-21-5p, miR-34a-5p and miR-155-3p, part of our miRNA panel, regulate the p53 pathway [42, 43]. As p53 aberrant IHC expression was not detected in NAFLD samples, we further focused on evaluating the expression of miRNAs that can alter their expression early on in tumorigenesis and can provide an insight on malignant transformation.

Based on the RT-PCR results (Fig. 8), miR-21-5p and miR-34a-5p maintained an upregulated expression pattern in both

peritumoral and tumoral non cirrhotic NAFLD-HCC when compared with the NAFLD samples. This confirms the findings obtained through our TCGA data analysis (Fig. 6). We also noticed a steady increase in the expression of both miR-21-5p and miR-34a-5p when comparing the peritumoral tissue samples with the tumoral ones. Hence, the upregulation of these two miRNAs is directly correlated with tumor development in our non-cirrhotic NAFLD-HCC patient cohort.

Hepatic-enriched miR-21-5p plays multiple oncometabolite roles in the NAFLD-HCC progression [44-46]. Its upregulated expression pattern found in our peritumoral and tumoral tissue samples was an expected event that confirms its value as an important molecular biomarker. Moreover, the implications of miR-21 in lipid accumulation in NAFLD and progression towards HCC have been shown by Wu et al., which demonstrated a mechanism of lipid accumulation via modulation of the HBP1-p53-Srebp1c pathway. Higher levels of miR-21-5p promoted lipid accumulation via downregulation of Hbp1, a transcriptional activator of the TP53 gene. Knockdown of miR-21 in mice models led to lower lipid accumulation and higher levels of HBP1 and p53 proteins, highlighting the inhibitory effect of miR-21 over the p53 pathway [47].

Likewise, the conserved upregulated expression profile of miR-34a-5p, known for its tumor suppressive roles in HCC [48, 49], deserves further attention regarding its value as a molecular biomarker. The miR-34a-5p is a tumor suppressor miRNA, usually down regulated in early tumorigenesis and associated with an aggressive phenotype [50]. Its expression was found to be increased in NAFLD [49, 51], which also explains its upregulation in NAFLD-HCC patients. Thus, miR-34a-5p appears to be associated with tumor progression, metastasis, and a negative outcome in HCC patients [52-54].

The miR-130a-3p is downregulated in the TCGA HCC samples (Fig. 6), and a similar result was obtained in our

cohort when comparing ANT with HCC samples. Interestingly, when comparing NAFLD samples with ANT, we observed an upregulation of the miR-130a-3p, which is innovative and supports the presence of a molecularly altered cancerized field in cancer patients. Also, we observed no significant changes when comparing NAFLD samples with HCC (Fig. 7 C). The miR-130a-3p modulates HCC proliferation and metastasis by targeting ZEB1/2 [55]. This miRNA was found to be downregulated in some studies on HCC and linked with prolonged overall survival. It is believed that miR-130a-3p may inhibit progression by downregulating ROCK2 [56]. Also, miR-130a-3p has been shown to downregulate the expression of β -catenin by targeting the WNT pathway, limiting proliferation and EMT [57].

The miR-155-3p was upregulated in the HCC samples when comparing NAFLD samples; no statistically significant differences were seen between ANT and HCC samples, but between NAFLD and ANT samples, we noticed a significant upregulation. Our results suggest the importance of evaluating the molecular aspects of the NAFLD tissue as it can reveal early-step molecular alterations. miR-155-3p is an important hepatic oncomiR, with multiple studies showing a correlation between its overexpression and the malignant progression of HCC [58]. MiR-155-3p was involved in multiple hallmarks of HCC, including tumorigenesis, cell proliferation, invasion, anti-apoptosis, and metastasis [59-61]. Studies have shown that miR-155-3p can target and downregulate the expression of p53, leading to decreased apoptosis, increased proliferation, and chemotherapy resistance in cancer cells [62, 63].

CONCLUSIONS

Our findings revealed molecular alteration of the expression profile of miRNAs in NAFLD tissue, especially for miR-21-5p, miR-34a-5p, and miR-130a-5p; their upregulation in peritumoral and tumoral tissue samples was associated with the progression of HCC in NAFLD patients.

In addition to their respective tumor suppressor and tumor-promoting roles, these 4 miRNAs play important roles in the progression of NAFLD to HCC by regulating the expression of key signaling pathways such as WNT pathway and influencing the expression of β -catenin and p53. The extensive study of molecular events inside hepatocytes will help understand the mechanism contributing to the development of HCC on a background of non-cirrhotic NAFLD and design new strategies to prevent, detect earlier, and develop new treatments for the disease.

Conflicts of interest: None to declare.

Authors' contribution: I.R., R.P. and I.B.N. conceived the study. R.P. and P.C. designed the methodology. I.R., V.R.P. and N.A. managed the samples. P.C. and R.O. used the specific software. P.C., L.B. and I.B.N. validated the results. C.B. and N.A. managed the patients. I.B.N. found the resources. I.R., R.P., P.C. and I.B.N. wrote the draft of the paper. C.B. and N.A. revised the manuscript. I.B.N. and N.A. supervised the study. All authors have read and agreed to the published version of the manuscript.

Acknowledgements: This paper was supported by the following projects: PDI-PFECIDI 2021, titled Increasing the Performance of Scientific Research, Supporting Excellence in Medical Research, and Innovation, PROGRES, no. 40PFE/30.12.2021 and SEE 21-COP-0049: Strategic inter-university cooperation to improve research abilities for Ph.D. students for higher educational quality. This paper was published under the frame of European Social Found, Human Capital Operational Program 2014-2020, project no. POCU/380/6/13/125171.

REFERENCES

1. Riazi K, Azhari H, Charette JH, et al. The prevalence and incidence of NAFLD worldwide: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol* 2022;7:851-861. doi:10.1016/S2468-1253(22)00165-0
2. Mantovani A, Byrne CD, Bonora E, Targher G. Nonalcoholic Fatty Liver Disease and Risk of Incident Type 2 Diabetes: A Meta-analysis. *Diabetes Care* 2018;41:372-382. doi:10.2337/dc17-1902
3. Loomis AK, Kabadi S, Preiss D, et al. Body Mass Index and Risk of Nonalcoholic Fatty Liver Disease: Two Electronic Health Record Prospective Studies. *J Clin Endocrinol Metab* 2016;101:945-952. doi:10.1210/jc.2015-3444
4. Huang DQ, El-Serag HB, Loomba R. Global epidemiology of NAFLD-related HCC: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol* 2021;18:223-238. doi:10.1038/s41575-020-00381-6
5. Global Burden of Disease Liver Cancer Collaboration; Akinyemiju T, Abera S, Ahmed M, et al. The Burden of Primary Liver Cancer and Underlying Etiologies From 1990 to 2015 at the Global, Regional, and National Level: Results From the Global Burden of Disease Study 2015. *JAMA Oncol* 2017;3:1683-1691. doi:10.1001/jamaoncol.2017.3055
6. Ioannou GN. Epidemiology and risk-stratification of NAFLD-associated HCC. *J Hepatol* 2021;75:1476-1484. doi:10.1016/j.jhep.2021.08.012
7. Kim D, Touros A, Kim WR. Nonalcoholic Fatty Liver Disease and Metabolic Syndrome. *Clin Liver Dis* 2018;22:133-140. doi:10.1016/j.cld.2017.08.010
8. Younossi ZM, Henry L. Epidemiology of non-alcoholic fatty liver disease and hepatocellular carcinoma. *JHEP Rep* 2021;3:100305. doi:10.1016/j.jhepr.2021.100305
9. Techathuvanan K, Srisajjakul S, Pongpaibul A, et al. Comparison between disease free survival of hepatocellular carcinoma after hepatic resection in chronic hepatitis B patients with or without cirrhosis. *J Med Assoc Thai* 2015;98:334-342.
10. Pinyopornpanish K, Khoudari G, Saleh MA, et al. Hepatocellular carcinoma in nonalcoholic fatty liver disease with or without cirrhosis: a population-based study. *BMC Gastroenterol* 2021;21:394. doi:10.1186/s12876-021-01978-0
11. Sherman M. Limitations of screening for hepatocellular carcinoma. *Hepat Oncol* 2014;1:161-163. doi:10.2217/hep.13.22
12. Zhou J, Sun H, Wang Z, et al. Guidelines for the Diagnosis and Treatment of Hepatocellular Carcinoma (2019 Edition). *Liver Cancer* 2020;9:682-720. doi:10.1159/000509424
13. Zhou H, Xu Q, Ni C, et al. Prospects of Noncoding RNAs in Hepatocellular Carcinoma. *Biomed Res Int* 2018;2018:6579436. doi:10.1155/2018/6579436
14. Yuan D, Chen Y, Li X, et al. Long Non-Coding RNAs: Potential Biomarkers and Targets for Hepatocellular Carcinoma Therapy and Diagnosis. *Int J Biol Sci* 2021;17:220-235. doi:10.7150/ijbs.50730

15. Xue C, Gu X, Bao Z, Su Y, Lu J, Li L. The Mechanism Underlying the ncRNA Dysregulation Pattern in Hepatocellular Carcinoma and Its Tumor Microenvironment. *Front Immunol* 2022;13:847728. doi:10.3389/fimmu.2022.847728
16. Deldar Abad Paskeh M, Mirzaei S, Ashrafzadeh M, Zarrabi A, Sethi G. Wnt/ β -Catenin Signaling as a Driver of Hepatocellular Carcinoma Progression: An Emphasis on Molecular Pathways. *J Hepatocell Carcinoma* 2021;8:1415-1444. doi:10.2147/JHC.S336858
17. Xu G, Xu WY, Xiao Y, et al. The emerging roles of non-coding competing endogenous RNA in hepatocellular carcinoma. *Cancer Cell Int* 2020;20:496. doi:10.1186/s12935-020-01581-5
18. Xu X, Tao Y, Shan L, et al. The Role of MicroRNAs in Hepatocellular Carcinoma. *J Cancer* 2018;9:3557-3569. doi:10.7150/jca.26350
19. Mei Y, You Y, Xia J, Gong JP, Wang YB. Identifying Differentially Expressed MicroRNAs Between Cirrhotic and Non-Cirrhotic Hepatocellular Carcinoma and Exploring Their Functions Using Bioinformatic Analysis. *Cell Physiol Biochem* 2018;48:1443-1456. doi:10.1159/000492254
20. Afonso MB, Rodrigues PM, Simão AL, Castro RE. Circulating microRNAs as Potential Biomarkers in Non-Alcoholic Fatty Liver Disease and Hepatocellular Carcinoma. *J Clin Med* 2016;5:30. doi:10.3390/jcm5030030
21. Dongiovanni P, Meroni M, Longo M, Fargion S, Fracanzani AL. miRNA Signature in NAFLD: A Turning Point for a Non-Invasive Diagnosis. *Int J Mol Sci* 2018;19:3966. doi:10.3390/ijms19123966
22. Rusu I, Pirlog R, Chiroi P, et al. The Implications of Noncoding RNAs in the Evolution and Progression of Nonalcoholic Fatty Liver Disease (NAFLD)-Related HCC. *Int J Mol Sci* 2022;23:12370. doi:10.3390/ijms232012370
23. Gjorgjieva M, Sobolewski C, Dolicka D, Correia De Sousa M, Foti M. miRNAs and NAFLD: from pathophysiology to therapy. *Gut* 2019;68:2065-2079. doi:10.1136/gutjnl-2018-318146
24. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;41:1313-1321. doi:10.1002/hep.20701
25. Moghaddam SJ, Haghighi EN, Samiee S, et al. Immunohistochemical analysis of p53, cyclinD1, RB1, c-fos and N-ras gene expression in hepatocellular carcinoma in Iran. *World J Gastroenterol* 2007;13:588-593. doi:10.3748/wjg.v13.i4.588
26. Vlachos IS, Zagganas K, Paraskevopoulou MD, et al. DIANA-miRPath v3.0: deciphering microRNA function with experimental support. *Nucleic Acids Res* 2015;43(W1):W460-W466. doi:10.1093/nar/gkv403
27. Li JH, Liu S, Zhou H, Qu LH, Yang JH. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res* 2014;42(Database issue):D92-D97. doi:10.1093/nar/gkt1248
28. Berindan-Neagoe I, Chiorean R, Braicu C, et al. Quantitative mRNA expression of genes involved in angiogenesis, coagulation and inflammation in multiforme glioblastoma tumoral tissue versus peritumoral brain tissue: lack of correlation with clinical data. *Eur Cytokine Netw* 2012;23:45-55. doi:10.1684/ecn.2012.0302
29. Cernea S, Onişor D. Screening and interventions to prevent nonalcoholic fatty liver disease/nonalcoholic steatohepatitis-associated hepatocellular carcinoma. *World J Gastroenterol* 2023;29:286-309. doi:10.3748/wjg.v29.i2.286
30. WenYi J, Ting Q, PiaoPiao Y, JinMing W. Association Between Neutrophil-to-Lymphocyte Ratio with Inflammatory Activity and Fibrosis in Non-alcoholic Fatty Liver Disease. *Turk J Gastroenterol* 2022;33:53-61. doi:10.5152/tjg.2022.20715
31. Thomas CE, Yu YC, Luu HN, et al. Neutrophil-lymphocyte ratio in relation to risk of hepatocellular carcinoma in patients with non-alcoholic fatty liver disease. *Cancer Med* 2023;12:3589-3600. doi:10.1002/cam4.5185
32. Anstee QM, Reeves HL, Kotsiliti E, Govaere O, Heikenwalder M. From NASH to HCC: current concepts and future challenges. *Nat Rev Gastroenterol Hepatol* 2019;16:411-428. doi:10.1038/s41575-019-0145-7
33. Refolo MG, Messa C, Guerra V, Carr BI, D'Alessandro R. Inflammatory Mechanisms of HCC Development. *Cancers (Basel)* 2020;12:641. doi:10.3390/cancers12030641
34. D'souza S, Lau KC, Coffin CS, Patel TR. Molecular mechanisms of viral hepatitis induced hepatocellular carcinoma. *World J Gastroenterol* 2020;26:5759-5783. doi:10.3748/wjg.v26.i38.5759
35. Kim E, Lisby A, Ma C, et al. Promotion of growth factor signaling as a critical function of β -catenin during HCC progression. *Nat Commun* 2019;10:1909. doi:10.1038/s41467-019-09780-z
36. Leung RWH, Lee TKW. Wnt/ β -Catenin Signaling as a Driver of Stemness and Metabolic Reprogramming in Hepatocellular Carcinoma. *Cancers (Basel)* 2022;14:5468. doi:10.3390/cancers14215468
37. Yemelyanova A, Vang R, Kshirsagar M, et al. Immunohistochemical staining patterns of p53 can serve as a surrogate marker for TP53 mutations in ovarian carcinoma: an immunohistochemical and nucleotide sequencing analysis. *Mod Pathol* 2011;24:1248-1253. doi:10.1038/modpathol.2011.85
38. Pirlog R, Chiroi P, Rusu I, et al. Cellular and Molecular Profiling of Tumor Microenvironment and Early-Stage Lung Cancer. *Int J Mol Sci* 2022;23:5346. doi:10.3390/ijms23105346
39. Link T, Iwakuma T. Roles of p53 in extrinsic factor-induced liver carcinogenesis. *Hepatoma Res* 2017;3:95-104. doi:10.20517/2394-5079.2017.07
40. Michelotti GA, Machado MV, Diehl AM. NAFLD, NASH and liver cancer. *Nat Rev Gastroenterol Hepatol* 2013;10:656-665. doi:10.1038/nrgastro.2013.183
41. Luo Z, Cui R, Tili E, Croce C. Friend or Foe: MicroRNAs in the p53 network. *Cancer Lett* 2018;419:96-102. doi:10.1016/j.canlet.2018.01.013
42. Hu B, Ma X, Fu P, et al. The mRNA-miRNA-lncRNA Regulatory Network and Factors Associated with Prognosis Prediction of Hepatocellular Carcinoma. *Genomics Proteomics Bioinformatics* 2021;19:913-925. doi:10.1016/j.gpb.2021.03.001
43. Wan Y, Cui R, Gu J, et al. Identification of Four Oxidative Stress-Responsive MicroRNAs, miR-34a-5p, miR-1915-3p, miR-638, and miR-150-3p, in Hepatocellular Carcinoma. *Oxid Med Cell Longev* 2017;2017:5189138. doi:10.1155/2017/5189138
44. Qu J, Yang J, Chen M, et al. MicroRNA-21 as a diagnostic marker for hepatocellular carcinoma: A systematic review and meta-analysis. *Pak J Med Sci* 2019;35:1466-1471. doi:10.12669/pjms.35.5.685
45. Hong Y, Ye M, Wang F, et al. MiR-21-3p Promotes Hepatocellular Carcinoma Progression via SMAD7/YAP1 Regulation. *Front Oncol* 2021;11:642030. doi:10.3389/fonc.2021.642030
46. Lai CY, Yeh KY, Lin CY, et al. MicroRNA-21 Plays Multiple Oncometabolic Roles in the Process of NAFLD-Related Hepatocellular Carcinoma via PI3K/AKT, TGF- β , and STAT3 Signaling. *Cancers (Basel)* 2021;13:940. doi:10.3390/cancers13050940
47. Wu H, Ng R, Chen X, Steer CJ, Song G. MicroRNA-21 is a potential link between non-alcoholic fatty liver disease and hepatocellular carcinoma via modulation of the HBP1-p53-Srebp1c pathway. *Gut* 2016;65:1850-1860. doi:10.1136/gutjnl-2014-308430

48. Niu X, Wei N, Peng L, Li X, Zhang X, Wang C. miR-34a-5p plays an inhibitory role in hepatocellular carcinoma by regulating target gene VEGFA. *Malays J Pathol* 2022;44:39-52
49. Ding J, Li M, Wan X, et al. Effect of miR-34a in regulating steatosis by targeting PPAR α expression in nonalcoholic fatty liver disease. *Sci Rep* 2015;5:13729. doi:[10.1038/srep13729](https://doi.org/10.1038/srep13729)
50. Wei W, Tang H, Tang L. MicroRNA-34a inhibits metastasis in liver cancer cells. *Oncol Lett* 2018;16:6960-6965. doi:[10.3892/ol.2018.9555](https://doi.org/10.3892/ol.2018.9555)
51. Yamada H, Suzuki K, Ichino N, et al. Associations between circulating microRNAs (miR-21, miR-34a, miR-122 and miR-451) and non-alcoholic fatty liver. *Clin Chim Acta* 2013;424:99-103. doi:[10.1016/j.cca.2013.05.021](https://doi.org/10.1016/j.cca.2013.05.021)
52. Zhou J, Zhou W, Kong F, Xiao X, Kuang H, Zhu Y. microRNA-34a overexpression inhibits cell migration and invasion via regulating SIRT1 in hepatocellular carcinoma. *Oncol Lett* 2017;14:6950-6954. doi:[10.3892/ol.2017.7090](https://doi.org/10.3892/ol.2017.7090)
53. Bernardi C, Soffientini U, Piacente F, Tonetti MG. Effects of microRNAs on fucosyltransferase 8 (FUT8) expression in hepatocarcinoma cells. *PLoS One* 2013;8:e76540. doi:[10.1371/journal.pone.0076540](https://doi.org/10.1371/journal.pone.0076540)
54. Chiu LY, Kishnani PS, Chuang TP, et al. Identification of differentially expressed microRNAs in human hepatocellular adenoma associated with type I glycogen storage disease: a potential utility as biomarkers. *J Gastroenterol* 2014;49:1274-1284. doi:[10.1007/s00535-013-0890-2](https://doi.org/10.1007/s00535-013-0890-2)
55. Li B, Huang P, Qiu J, Liao Y, Hong J, Yuan Y. MicroRNA-130a is down-regulated in hepatocellular carcinoma and associates with poor prognosis. *Med Oncol* 2014;31:230. doi:[10.1007/s12032-014-0230-2](https://doi.org/10.1007/s12032-014-0230-2)
56. Zheng Y, Xiang L, Chen M, Xiang C. MicroRNA130a inhibits the proliferation, migration and invasive ability of hepatocellular carcinoma cells by downregulating Rhokinase 2. *Mol Med Rep* 2018;18:3077-3084. doi:[10.3892/mmr.2018.9283](https://doi.org/10.3892/mmr.2018.9283)
57. Poodineh J, Sirati-Sabet M, Rajabibazl M, Mohammadi-Yeganeh S. MiR-130a-3p blocks Wnt signaling cascade in the triple-negative breast cancer by targeting the key players at multiple points. *Heliyon* 2020;6:e05434. doi:[10.1016/j.heliyon.2020.e05434](https://doi.org/10.1016/j.heliyon.2020.e05434)
58. Fu X, Wen H, Jing L, et al. MicroRNA-155-5p promotes hepatocellular carcinoma progression by suppressing PTEN through the PI3K/Akt pathway. *Cancer Sci* 2017;108:620-631. doi:[10.1111/cas.13177](https://doi.org/10.1111/cas.13177)
59. Ning S, Liu H, Gao B, et al. miR-155, miR-96 and miR-99a as potential diagnostic and prognostic tools for the clinical management of hepatocellular carcinoma. *Oncol Lett* 2019;18:3381-3387. doi:[10.3892/ol.2019.10606](https://doi.org/10.3892/ol.2019.10606)
60. Chen G, Wang D, Zhao X, et al. MiR-155-5p modulates malignant behaviors of hepatocellular carcinoma by directly targeting CTHRC1 and indirectly regulating GSK-3 β -involved Wnt/ β -catenin signaling. *Cancer Cell Int* 2017;17:118. doi:[10.1186/s12935-017-0469-8](https://doi.org/10.1186/s12935-017-0469-8)
61. Tang B, Lei B, Qi G, et al. MicroRNA-155-3p promotes hepatocellular carcinoma formation by suppressing FBXW7 expression. *J Exp Clin Cancer Res* 2016;35:93. doi:[10.1186/s13046-016-0371-6](https://doi.org/10.1186/s13046-016-0371-6)
62. Van Roosbroeck K, Fanini F, Setoyama T, et al. Combining Anti-Mir-155 with Chemotherapy for the Treatment of Lung Cancers. *Clin Cancer Res* 2017;23:2891-2904. doi:[10.1158/1078-0432.CCR-16-1025](https://doi.org/10.1158/1078-0432.CCR-16-1025)
63. Gironella M, Seux M, Xie MJ, et al. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. *Proc Natl Acad Sci U S A* 2007;104:16170-16175. doi:[10.1073/pnas.0703942104](https://doi.org/10.1073/pnas.0703942104)