Differential expression of angiogenesis-related miRNAs and *VEGFA* in cirrhosis and hepatocellular carcinoma

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Abstract

Introduction: Liver cirrhosis (LC) is a heterogeneous liver disease, the last stage of liver fibrosis, and the major risk factor for hepatocellular carcinoma (HCC). Our study aimed to evaluate the expression of microRNAs and the endothelial vascular growth factor (*VEGFA*) gene in LC and HCC.
Material and methods: The sample group consisted of 46 tissue samples: 21 of LC, 15 of HCC, and 10 of non-tumoural and non-cirrhotic liver tissue (control group). MiRNAs were chosen based on a mirDIP prediction database as regulators of the *VEGFA* gene. Gene expression of VEGF and miRNAs was quantified by real-time quantitative polymerase chain reaction. VEGFA protein expression was evaluated by ELISA.
Results: *VEGFA* gene expression was significantly overexpressed in LC compared to the control group ($p < 0.0001$). Hsa-miR-206 ($p = 0.0313$) and hsa-miR-637 ($p = 0.0156$) were down-expressed in LC. In HCC, hsa-miR-15b ($p = 0.0010$), hsa-miR-125b ($p = 0.0010$), hsa-miR-423-3p ($p = 0.0010$), hsa-miR-424 ($p = 0.0313$), hsa-miR-494 ($p < 0.0001$), hsa-miR-497 ($p < 0.0001$), hsa-miR-612 ($p = 0.0078$), hsa-miR-637 ($p < 0.0001$), and hsa-miR-1255b ($p = 0.0156$) presented down-expression.
Conclusions: Overexpression of *VEGFA* in LC suggests impairment of angiogenesis in this tissue. The differential expression of microRNAs in LC and HCC observed in our study can lead to the evaluation of possible biomarkers for these diseases.

Key words: liver diseases, *VEGFA*, microRNAs, biomarkers.

Introduction

Liver cirrhosis (LC) is a heterogeneous liver disease, the last stage of liver fibrosis, and the major risk factor for hepatocellular carcinoma (HCC). The major risk factors for LC are hepatitis B and C (HBV and HCV, respectively), alcoholic liver disease, and non-alcoholic steatohepatitis (NASH). LC is the major risk factor for HCC. HCC is the sixth most common type of cancer worldwide and the second leading cause of death, with high malignancy. LC is defined by regeneration nodules involved in fibrosis and the healing process, which lead to substitution of hepatic tissue and function loss due to chronic injury of the liver [1, 2]. Compli-

cations associated with this disease are ascites, oesophageal varices, and encephalopathy. Prognosis is related to disease staging as assessed by standard scales such as the model for end-stage liver (MELD) and Child-Pugh. Treatment consists of alcohol abstinence, a specific diet, and liver transplantation [3, 4].

HCC is more frequent in developing countries, where 83% of cases occurred in Asia in 2012 [5]. LC, alcohol abuse, aflatoxins exposure, HBV and HCV infections, autoimmune hepatitis (AIH), and NASH are important risk factors for HCC development [6–8]. Prevention is based on HBV vaccination and the use of agents that reduce carcinogenesis in patients with a higher chance of developing the disease [9].

HCC tissue is highly vascularized; therefore, the angiogenic process represents an important role in the formation, proliferation, and metastasis of the tumour. The *VEGFA* gene is responsible for translating vascular endothelial growth factor A, which is an important mitogen of endothelial cells and acts on new vessel formation in the tumour [10]. Studies report that *VEGFA* expression is increased in HCC, and most types of cancer express this growth factor. Furthermore, VEGFA presents oncogenicity once it increases cell proliferation [11, 12].

In addition, post-transcriptional regulatory processes are performed by non-coding RNAs, microRNAs (miRNA, miR), which are single-stranded molecules of approximately 22 nucleotides in length. MiRNA is responsible for inhibiting protein translation by binding to target messenger RNA (mRNA), leading to degradation or only inhibition. Changes in gene expression of regulatory factors may reduce or elevate the expression of target genes and lead to tumour development as well as its growth and proliferation [13–15]. MiRNA regulation is modified by changes in gene expression, and imbalances may increase carcinogenesis in tissues. MiRNAs regulate the *VEGFA* gene in tissues, and alteration in this pathway is related to pathogenicity and growth of injured tissues [16]. Therefore, the aim of this study was to evaluate the expression of the *VEGFA* gene and 17 miRNAs predicted to be regulators of *VEGFA* in LC and HCC samples.

Material and methods

Samples

The sample group consisted of 46 diagnosticated tissue samples: 21 of LC, 15 of HCC, and 10 of non-tumoural and non-cirrhotic liver tissue (control group). These samples were collected in videolaparoscopic hepatectomy, biopsy, and liver transplantation procedures performed at the Sur-

gical Centre of Hospital de Base de São José do Rio Preto (FAMERP/FUNFARME) after sample donors signed the informed consent form (previously approved by the Research Ethics Committee, CEP/FAMERP, no. 6228/2011). After collection, these samples were stored in RNAlater (Thermo Fisher Scientific, Carlsbad, CA) according to the manufacturer's instructions and stored at –20ºC until RNA and protein isolation.

In silico miRNA analysis

MiRNAs were chosen based on a mirDIP prediction database (ophid.utoronto.ca/mirDIP/). Seventeen miRNAs were selected (hsa-miR-1, hsa-miR-15b, hsa-miR-125b, hsa-miR-206, hsa-miR-423-3p, hsa-miR-424, hsa-miR-452, hsa-miR-494, hsa-miR-497, hsa-miR-576-5p, hsa-miR-612, hsa-miR-637, hsa-miR-874, hsa-miR-889, hsa-miR-920, hsa-miR-1255a, hsa-mir-1255b) that were predicted to be regulators of the *VEGFA* gene. Selected miRNAs presented the maximum score for prediction at choice time.

Total RNA and protein isolation

Total RNA and protein were isolated using the mirVana PARIS kit (Thermo Fisher Scientific, Carlsbad, CA) according to the manufacturer's instructions. Total RNA quantification was performed using Picodrop P200 (Picodrop Limited, Cambridgeshire, UK), and 230/280 and 260/280 ratios were measured to evaluate purity and precipitated salt contamination, respectively. Total protein of samples was quantified by the BCA Protein Assay Method (Pierce, Rockford, USA). During isolation and quantification, samples were kept in ice to avoid degradation and stored at –80ºC.

Reverse transcription polymerase chain reaction

Total RNA was reverse-transcribed for *VEGFA* gene expression analysis using a high capacity reverse transcription kit (Thermo Fisher Scientific, Carlsbad, CA) according to the manufacturer's protocol. A TaqMan miRNA Reverse Transcription kit (Thermo Fisher Scientific, Carlsbad, CA) was used to evaluate miRNA expression, according to the manufacturer's protocol.

RT-qPCR

The analysis of *VEGFA* gene expression was performed using the TaqMan Gene Expression Assay (Hs00900055_m1) (Thermo Fisher Scientific, Carlsbad, CA). Gene expression data were normalized using *RPLPO, GAPDH,* and *HPRT1* as reference genes. The TaqMan MicroRNA Custom Assay (Thermo Fisher Scientific, Carlsbad, CA)

Table I. Characteristics of LC and HCC patients

Variables	Sample groups	
	LC	HCC
Age (median)	59	58
Alcohol consumption	56.52%	53.34%
Tobacco consumption	56.52%	40%
HBV	26.08%	20%
HCV	43.47%	33.34%
AIH	4,34%	13.34%
Liver cirrhosis	–	93.34%

AIH – autoimmune hepatitis, HBV – hepatitis B virus, HCV – hepatitis C virus.

was used to analyse miRNA expression. The assay contained primers and probes for the 17 miRNAs predicted to be *VEGFA* regulators. U6, RNU44, and RNU48 were used as reference genes for miRNA analysis. Relative quantification (RQ) was calculated in relation to non-tumoural and non-cirrhotic liver tissue (calibrator) using the $2^{-\Delta\Delta Ct}$ method [17]. The reactions were performed on a StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA) and cycled following the manufacturer's instructions.

VEGFA protein quantification

VEGFA protein quantification was performed by enzyme-linked immunosorbent assay (ELISA), using the ELISA Duo Set (R&D Systems, Minneapolis,

Table II. Expression of *VEGFA* gene according to tumour size

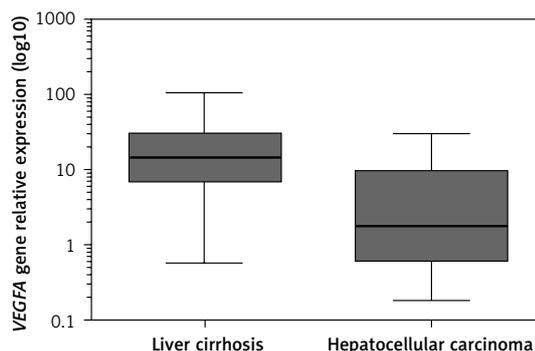| Sample	Tumour size	RQ
T1	1.2	20.79
T2	1.3	9.71
T3	1.4	30.80
T4	1.4	1.34
T5	1.4	12.88
T6	2.0	0.57
T7	2.0	0.71
T8	2.1	0.60
T9	2.5	0.79
T10	2.8	1.81
T11	3.1	5.06
T12	3.8	0.18
T13	4.3	1.89
T14	5.0	2.02
T15	8.5	0.31

Figure 1. Representative RQ median of *VEGFA* gene expression in liver cirrhosis and hepatocellular carcinoma tissues

USA) according to the manufacturer's instructions. Optical density was determined using a reader set to 450 nm (Thermo Fisher Scientific, Carlsbad, CA) using SkanIt Software (Thermo Fisher Scientific, Carlsbad, CA). The reactions were duplicated.

Statistical analysis

Statistical tests were performed using Graph-Pad Prism 6. The d'Agostino and Pearson normality test was used to test the data for normal distribution. Grubbs' outliers test was performed to check the normally distributed data for outliers. A *t*-test, Wilcoxon test, and Mann-Whitney test were performed for comparisons between the groups. *P*-values of < 0.05 were considered significant.

Results

Characterization of LC and HCC groups is presented in Table I. The variables evaluated included age, alcohol and tobacco consumption, HBV and HCV virus infections, and autoimmune hepatitis (AIH).

Analysis of *VEGFA* gene expression showed significant overexpression in LC compared to the control group, with RQ median of 14.55 ($p < 0.0001$). Nine samples of HCC presented high expression of *VEGFA* (56.25%) in relation to the control group but without statistical significance (RQ median = 1.874, $p = 0.1040$). *VEGFA* gene expression was 12.97-fold higher in LC than in HCC ($p = 0.0001$) (Figure 1).

VEGFA gene expression was negatively correlated with tumour size ($R = -0.52$, $p = 0.048$). Tumours smaller than 2.1 cm (median) presented high expression of *VEGFA*, while tumours larger than 2.1 cm presented low expression (Table II).

In relation to miRNAs, 15 miRNAs showed expression in both sample tissues. Hsa-miR-206 ($p = 0.0313$) and hsa-miR-637 ($p = 0.0156$) were down-expressed in LC (Figure 2). In HCC, hsa-miR-15b ($p = 0.0010$), hsa-miR-125b ($p = 0.0010$), hsa-miR-423-3p ($p = 0.0010$), hsa-miR-424 ($p = 0.0313$), hsa-miR-494 ($p < 0.0001$), hsa-miR-497
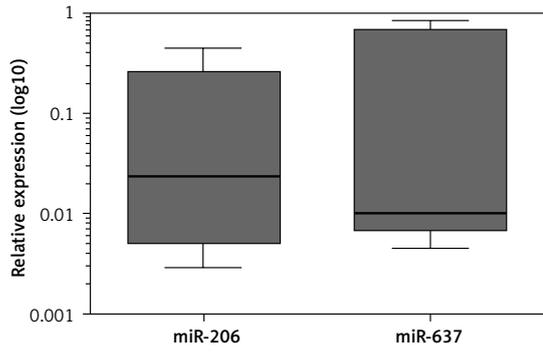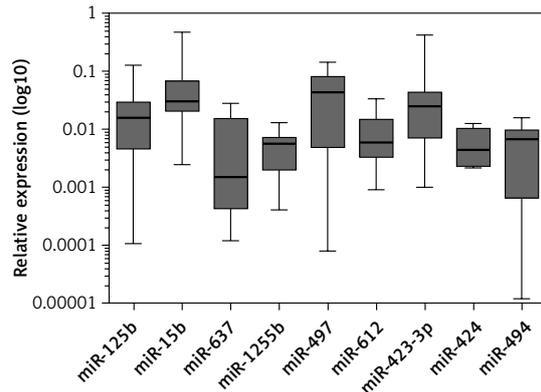
Figure 2. Differential expression of miRNAs in LC

($p < 0.0001$), hsa-miR-612 ($p = 0.0078$), hsa-miR-637 ($p < 0.0001$), and hsa-miR-1255b ($p = 0.0156$) presented down-expression (Figure 3, Table III).

Regarding protein expression, there was no significant difference in *VEGFA* expression between HCC and the control group ($p = 0.9999$) or between LC and the control group ($p = 0.1309$). However, *VEGFA* expression was higher in LC than in HCC (77.04 µg/ml vs. 54.16 µg/ml, respectively; $p = 0.0078$). Therefore, LC tissues presented *VEGFA* protein expression 0.4-fold higher than tumour samples.

Discussion

VEGFA is the most important angiogenic growth factor in physiological and pathological states. Increased expression of *VEGFA* is observed in a variety of solid tumours [10, 18–20], including HCC [21, 22]. HCC samples presented high expression of *VEGFA* in relation to the control group in

Figure 3. Differential expression of miRNAs in HCC

our study but without statistical significance. In LC samples *VEGFA* was significantly overexpressed. Ninety-three percent of HCC patients have cirrhosis, which is a pre-cancer stage and represents the main risk factor for this tumour type [23].

High expression of *VEGFA* in LC samples indicates the presence of angiogenic factors that contribute to new vessel formation and substitution of liver tissue for fibrous tissue [24, 25]. Campagnolo *et al.* [22] observed that *VEGFA* and *EGFL7* gene expression levels were 120-fold and 25-fold higher, respectively, in LC tissue samples compared to normal tissue [22]. Von Marschall *et al.* [26] found *VEGFA* expression in 73% of LC tissue samples, and this increase may be related to the formation of fibrous tissue (necrotic foci), which secretes *VEGFA*. Yang *et al.* [27] verified that high expression of *VEGFA* was associated with fast tissue repair and more fibrogenic activity. El-Assal

Table III. miRNAs differentially expressed in LC and HCC

miRNA	Tissue sample	Median or mean	*P*-value	Function related	Detected expression	Reference
hsa-miR-206	LC	0.02369	0.0313	Cell proliferation suppression, apoptosis	Low	[35, 36]
hsa-miR-637	LC and HCC	0.01019 and 0.006758	0.0156 and < 0.0001	LIF gene inhibition, induced STAT3 phosphorylation	Low	[33]
hsa-miR-15b	HCC	0.03091	0.0010	Apoptosis, proliferation, survival and angiogenesis	Low	[37, 38]
hsa-miR-125b	HCC	0.01632	0.0010	Cell invasion, tumour metastasis, VEGF regulation	Low	[37, 42]
hsa-miR-423-3p	HCC	0.02558	0.0010	Cell proliferation, cell cycle	Low	[46, 48]
hsa-miR-424	HCC	0.004519	0.0313	Angiogenesis regulation and cell invasion	Low	[51, 53]
hsa-miR-494	HCC	0.006319	< 0.0001	Cell cycle, sorafenib resistance, PTEN regulation	Low	[59]
hsa-miR-497	HCC	0.04621	< 0.0001	Invasion, proliferation, cell growth and apoptosis	Low	[63–66]
hsa-miR-612	HCC	0.00605	0.0078	Metastasis suppressor	Low	[69]
hsa-miR-1255b	HCC	0.00568	0.0156	No functional study in the literature	Low	–

et al. [28] also observed a significantly high expression in LC tissue (RQ mean = 56.4) in relation to control (RQ mean = 42.0). All studies confirm our findings.

We observed that tumours larger than 2.1 cm presented down-expression of the *VEGFA* gene. Yamaguchi *et al.* [29] showed that tumours larger than 3 cm down-expressed *VEGFA*. In HCC, tumours larger than 5 cm presented fewer blood vessels than smaller tumours [28–30]. This inverse relationship may occur because small tumours require the formation of new blood vessels for growth, while large tumours may secrete other growth factors such as TGF-β, bFGF, TNF-α, and IL-8 [25].

The *VEGFA* gene is regulated at all stages of gene expression. Numerous studies have identified miRNAs targeting *VEGFA* [31]. We performed extensive research on miRNA databases and selected 17 miRNAs for investigation in LC and HCC samples. These miRNAs were predicted to be regulators of *VEGFA* gene expression according to the maximum score on prediction.

In both samples, HCC and LC, hsa-miR-637 presented reduced expression. This miRNA was found at reduced levels in HCC cell lines, and its increase resulted in cell death, whereas its suppression reduced the apoptosis rate to 8%. These results suggest that hsa-miR-637 plays a protective role in HCC and cirrhosis. It was verified that hsa-miR-637 is responsible for the inhibition of the *LIF* (leukaemia inhibitory factor) gene, which translates a cytokine responsible for inducing Stat3 phosphorylation. This transcription factor regulates several growth factors, including the *VEGFA* gene [32]. Reduced expression of this miRNA in glioma cells was associated with a poor prognosis by promoting growth, migration, and invasion of these cells [33]. Reduced expression of hsa-miR-637 may be related to tumour initiation in LC patients. It is known that LC is the major risk factor for HCC and a pre-cancer stage.

Hsa-miR-206 was down-regulated in LC in this study. In HCC samples, this miRNA did not show differential expression. This miRNA was found differentially expressed in LC and HCC in a previous study but was more highly expressed in HCC than in LC [34]. On the other hand, it was observed at low levels in HCC and was related to low tumour differentiation [35]. The increase of hsa-miR-206 levels in HCC cell lines resulted in suppression of cell proliferation, invasion, and migration and promoted apoptosis [35]. Overexpression of miR-206 was associated with promotion of apoptosis, induction of the cell cycle, and inhibition of migration in HepG2 cells [36]. Therefore, the role of this miRNA is not completely elucidated in cirrhosis and HCC.

In addition to hsa-miR-637, HCC samples also presented down-expression of hsa-miR-15b, hsa-miR-125b, hsa-miR-423-3p, hsa-miR-424, hsa-miR-

494, hsa-miR-497, hsa-miR-612, and hsa-miR-1255b. In acute liver failure, the reduction of hsa-miR-15b levels led to a decrease of apoptosis and proliferation and survival of tumour cells [37]. In HCC, this miRNA was down-expressed and was related to tumour proliferation [38]. Furthermore, the high expression of this miRNA reduced HCC recurrence after resection, showing that it could be an important prognostic marker for this tumour type [38]. It was observed that hsa-miR-15b regulated the *VEGFA* gene in nasopharyngeal carcinoma in hypoxic conditions [39, 40]. In glioma, miR-15b was responsible for reduction of angiogenesis, targeting other genes (*NRP-2* and *MMP-3*) [41].

As in our study, hsa-miR-125b was down-expressed in HCC in other studies [37, 42]. This miRNA was responsible for repression of cell invasion and tumour metastasis by preventing the formation of blood vessels [43]. Therefore, the down-expression observed in our study corroborates the hypothesis that hsa-miR-125b could play a role in the protection of hepatic tissue. The relation of the *VEGFA* gene and this miRNA was found in recovery of denatured dermis, where this miRNA presented reduced levels after the third day of burning, increasing *VEGFA* gene expression for new vessel formation for reconstitution of injured tissue [44]. In rats, low levels of this miRNA were observed in biliary ducts and were associated with the *HDC* gene (L-histamine decarboxylase), which is a down-regulator of *VEGFA*, influencing its expression in biliary synthesis [45].

High expression of hsa-miR-423-3p increased cell proliferation in breast cancer cell lines [46], laryngeal carcinoma [47], and HCC [48]. In HCC cells, miR-423 promoted cell growth and cell cycle progression at the G1/S transition [48]. This miRNA was considered a new oncogenic miRNA in HCC because of its role in suppression of the tumour suppressor *p21Cip1/ Waf1* [48]. In colorectal cancer, this miRNA was found at high expression levels and promoted cell cycle progression [49]. The overexpression of miR-423-3p in several cancer types suggests a role in tumour development and progression. We observed down-expression of miR-423-3p in HCC. Our results suggest that this miRNA did not play an essential role in tumour promotion in our study and should be thoroughly investigated in HCC.

Hsa-miR-424 was related to the regulation of angiogenesis in endothelial cells, targeting the *VEGFR2* gene [50]. Corroborating our results, this miRNA was observed in down-expression in HCC [51, 52], and these levels of hsa-miR-424 had no influence on the suppression of cell migration and invasion, contributing to tumour development [51]. Another study demonstrated that the survival rate of HCC patients is lower in those with

low serum levels of this miRNA [53]. Hsa-miR-424 was associated with the lysosome pathway and the D-glutamine and D-glutamate metabolism pathway. The lysosome pathway is linked to cell metabolism, cell growth, dead cell clearance, and ubiquitination [52]. Impaired lysosomal maturation can contribute to the carcinogenesis of HBV-related HCC [36]. The down-expression of this miRNA is responsible for acquisition and subsequent maintenance of the tumour stem cell phenotype via the target *PBX3* gene, enabling HCC cells to reprogramme into tumour stem cells [54]. Furthermore, overexpression of miR-424 represses the cell cycle/E2F pathway by targeting Akt3 and E2F3, resulting in inhibition of HCC growth and an increase of the survival rate after liver resection [55]. In addition, down-regulation of miR-424 results in increased expression of c-Myb, a pro-invasive molecule that contributes to malignant transformation, cell growth, angiogenesis, and resistance to apoptosis [56–58].

Hsa-miR-494 regulates the cell cycle in HCC, and its suppression reduced cell transformation. It was also observed that in high expression, miR-494 contributed to cell proliferation and increased resistance to sorafenib. A study revealed that the suppression of the *PTEN* gene by this miRNA increased proliferation, invasion, and migration of HCC cells [59]. Up regulation of miRNA-494 has been identified as a potential non-invasive biomarker for HCC until now [60–62], although we found down-regulation of this miRNA in our HCC samples. Therefore, further studies are needed to clarify the role of miRNA-494 in HCC.

In HCC and breast cancer, hsa-miR-497 showed low expression and was related to apoptosis and the suppression of invasion, proliferation, and cell growth [63]. This miRNA was suggested to be an angiogenesis inhibitor in HCC, directly targeting the *VEGFA* gene, as well as in ovarian cancer [63–66]. Moreover, miR-497 led to significant up-regulation of CHEK1, a Ser/Thr kinase that mediates cell-cycle arrest after DNA damage. CHEK1 overexpression resulting from the down-regulation of miR-497 in HCC is responsible for tumour development and poor prognosis [67]. Our result regarding this miRNA corroborates this hypothesis.

In our study, hsa-miR-612 was down-expressed in HCC. This result corroborates a previous study that observed inhibitory effects of this miRNA on cell proliferation, migration, invasion, and metastasis of HCC [68]. MiR-612 was able to suppress the stemness of HCC via Wnt/b-catenin signalling, reduce the number and size of tumourspheres, and prevent the capacity of tumourigenesis in NOD/SCID mice [68]. Furthermore, miR-612 was observed to have a role in suppression of HCC stemness via the Sp1/Nanog signalling pathway [69]. MiR-612 inhibited the protein level of Sp1

and reduced the transcription activity of Nanog in a recent study. Nanog is a transcription factor in embryonic stem cells (ESCs) and is thought to play a critical role in maintenance of pluripotency [70].

Regarding hsa-miR-1255b, there are no data in the literature about functional studies with this miRNA. The expression levels of miR-1255b have not been examined so far in cancer cells. The down-expression of this miRNA in HCC samples in our study suggests a possible role in liver tumourigenesis and should be investigated in further studies as a novel biomarker of HCC.

In addition to *VEGFA* and miRNAs, other biomarkers have been used for the identification of cellular and genetic alterations and neoplasms as well as the prognosis and diagnosis of diseases, such as α-fetoprotein (a serologic marker) [71], cyclooxygenase-2 (COX-2, an important enzyme in carcinogenesis in HCC) [72], B-catenin and E- and N-cadherins (involved in cellular alterations and indicators of epithelial-mesenchymal transition) [73], vitamin D deficiency (mainly in women with cardiovascular risk) [74], and presenilin 2 (*PSEN2,* a possible tumour suppressor in renal cancer) [75].

In conclusion, our results showed overexpression of the *VEGFA* gene in LC and down-expression of miRNAs in LC and HCC. These miRNAs are involved in important pathways for cancer development. It is important to emphasize that these miRNAs could be potential biomarkers for LC and HCC, which can direct the treatment of these diseases. The differential expression of mR-1255b was identified for the first time in HCC and suggests a novel biomarker for this tumour type. The discovery of biomarkers is relevant for diagnosis and prognosis of these diseases. Functional studies involving miRNAs and target genes can help in the development of new adjuvant therapies in LC and HCC treatment.

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Conflict of interest

The authors declare no conflict of interest.

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