miRNA Signature of Hepatocellular Carcinoma Vascularization:
How the Controls Can Influence the Signature

Silvia Fittipaldi1 · Francesco Vasuri1 · Sonia Bonora1 · Alessio Degiovanni1 · Giacomo Santandrea1 · Alessandro Cucchetti2 · Laura Gramantieri3 · Luigi Bolondi3 · Antonia D'Errico1

Received: 29 March 2017 / Accepted: 13 June 2017 / Published online: 21 June 2017 © Springer Science+Business Media, LLC 2017

Abstract
Background miRNA deregulation and vascular modifications constitute promising predictors in the study of hepatocellular carcinoma (HCC). In the literature, the relative miRNA abundance in HCC is usually determined using as control non-matched tumoral tissue, healthy liver, or cirrhotic liver. However, a common standard RNA control for the normalization toward the tissue gene expression was not settled yet.
Aim To assess the differences existing in the quantitative miRNA gene expression in HCC on tissue according to two different liver controls.
Methods A wide array of miRNAs was analyzed on 22 HCCs arisen in cirrhotic and non-cirrhotic livers by means of microfluidic cards. Control samples included total RNA extracted from healthy and cirrhotic livers.
Results Six miRNAs were deregulated in HCCs using either controls: miR-532, miR-34a, miR-93, miR-149#, miR-7f-2#, and miR-30a-5p. Notably, the miRNA expression changed significantly between HCCs arisen in cirrhotic and non-cirrhotic livers, according to the control used for normalization. Different miRNA profiles were found also in HCCs with different vascular patterns, according to the control used for normalization.
Conclusions Our data confirm that the choice of the methodology, and particularly the control used for normalization, represents the main concern in miRNA evaluation, particularly in a heterogeneous model such as liver pathology. Still we observed the deregulation of some common miRNAs as promising in HCC cancerogenesis and progression. A standardized control will be a crucial achievement to compare miRNA expression among different laboratories.

Electronic supplementary material The online version of this article (doi:10.1007/s10620-017-4654-3) contains supplementary material, which is available to authorized users.

Francesco Vasuri
vasurifrancesco@libero.it
Silvia Fittipaldi
sv.fittipaldi@gmail.com
Sonia Bonora
soniabonora@hotmail.it
Alessio Degiovanni
alessiodegiovanni@gmail.com
Giacomo Santandrea
giacomo.santandrea@studio.unibo.it
Alessandro Cucchetti
alessandro.cucchetti@aosp.bo.it
Laura Gramantieri
laura.gramantieri@aosp.bo.it

1 “F. Addarri” Institute of Oncology and Transplant Pathology, Department of Specialty, Diagnostic and Experimental Medicine (DIMES), S. Orsola-Malpighi University Hospital, V.le Ercolani 4/2, 40138 Bologna, Italy
2 Department of Medical and Surgical Sciences (DIMEC), S. Orsola-Malpighi University Hospital, Bologna, Italy
3 Division of Internal Medicine, Department of Medical and Surgical Sciences, University of Bologna, S. Orsola-Malpighi University Hospital, Bologna, Italy
Keywords Cirrhosis · Hepatocellular carcinoma · Microfluidic card · miRNA · Neoangiogenesis

Introduction

Liver resection represents the therapeutic choice for hepatocellular carcinomas (HCCs) without concomitant liver failure. Although post-resection outcome shows substantial differences, best results are seen in Child-Pugh class A patients with small lesions without microvascular invasion (MVI) or metastases and with free surgical margins [1]. The predictors of HCC recurrence are mainly based on clinical or morphological features, and new research lines are strongly needed in order to find and validate new prognostic markers. The study of miRNAs (small RNAs characterized by regulatory functions on gene expression that act as gene promoters or suppressors) represents a promising approach toward further understanding of human carcinogenesis and tumor progression [2]. Another important approach is the analysis of neoangiogenesis and vascular modifications in HCCs. Villa et al. [3] found five genes involved in neoangiogenesis and/or endothelial activation to be up-regulated in fast-growing HCCs. Our group performed immunohistochemical and gene expression analysis on HCC vascular modifications. We found four different vascular patterns in HCCs, each of them characterized by different tumor architecture, vascular morphology and phenotype, as well as by different expression of Nestin, IGF1R and TGF-β1 [4].

In HCCs, the biosynthesis pathways of different miRNAs were found to promote neoangiogenesis, tumor invasion and metastasization at different levels, from transcriptional (e.g., down-regulation of miR-122) [5] and post-transcriptional regulations (e.g., miR-99 and miR-21) [6, 7], up to epigenetic and genetic changes [8].

In miRNA studies, choice of methodology is of utmost importance. Several studies focused on the normalization of gene expression toward reference genes [9–11]. However, methodological studies using a standard for normalization toward tissue gene expression are needed. Hitherto, research has focused on the importance of the reference genes but not on the appropriateness of the control tissues [9]. Despite the high number of papers about miRNA, shared criteria for standardizing the methodology for the quantification have not been established yet. There is also lack of details on the sample source, the RNA isolation, and the control tissues treatment and origin [12]. In the last decade, some miRNA studies have been carried on with arrays on human HCC and non-neoplastic tissue, and most of them utilized adjacent liver tissue for the normalization [6, 13, 14]. With this approach, Budhu et al. [14] found 20 miRNAs significantly associated with metastases, but only one miRNA overlapped with the results obtained by Murakami et al. [13]. In a more recent paper by Sato et al., healthy liver tissue was used for the normalization in the analysis of both HCC and non-neoplastic tissue. Authors found that different miRNAs were deregulated in both HCCs and non-neoplastic livers and significantly correlated with the tumor recurrence after transplantation [15]. Another methodology used the matched non-neoplastic tissue as control for each HCC, this method is useful to minimize the individual genetic variability, but it is expensive and prevents the identification of a universal control RNA with intralaboratory reproducibility. [16–18]. Thus, according to the goals of the studies, different controls were selected to assess differential miRNA expression.

The choice of the control tissues as reference for normalization in each analysis is the key point, since it was demonstrated that results can vary widely with different reference controls, and a single miRNA can turn out to be down-regulated in an experiment and up-regulated in another, just by changing the control [19, 20]. The topic is even more tangled in the study of liver tumors, where the control tissue is seldom represented by “healthy” liver, since most HCCs arise in a cirrhosis or in a chronic hepatitis. In the perspective of a miRNA-regulatory therapy, this could be a challenge [20].

We hereby will demonstrate the concern and the differences that emerged by using two different control tissues to normalize miRNA gene expression in HCC. These two controls represent the opposite biological backgrounds in HCCs pathogenesis; total RNA was extracted from virus-free and cancer-free healthy livers, as well as from HCV-related cirrhotic livers. The different miRNA profiles of resectable HCCs arisen in cirrhotic (c-HCC) and non-cirrhotic (nc-HCC) livers will be determined. Finally, miRNA expression will be correlated with the patterns of vascular modifications of HCCs, in order to confirm the existence of different steps in HCC progression. The identification of pattern-specific miRNAs possibly correlated with neoangiogenesis and tumor architecture could contribute to better understand HCCs behavior.

Methods

Ethics

The present tissue study was approved in advance by the Ethical Committee of the S. Orsola-Malpighi University Hospital (protocol code APHCC-2012, reference number 85/2013/O/Tess). All patients were treated according to the...
ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008); informed consent was obtained from each patient at the time of surgery.

Case Selection, Histopathology and Immunohistochemistry

We retrospectively selected 22 consecutive patients submitted to surgical resection for HCC. Patients were 19 (86.4%) males and 3 (13.6%) females, mean age at surgery 62.9 ± 9.9 years (range 48–80 years). The etiology of cirrhosis or chronic liver disease was HCV in 12 cases, HBV in 5, metabolic in 2, alcoholic in 1, and cryptogenic in 2.

Tissue was promptly fixed in formaldehyde solution 4% directly in surgery room and buffered pH 6.9 before routine processing. For each tumor, we evaluated the occurrence of cirrhosis, the mean tumor size, Edmondson’s grade, the occurrence of infiltrative margins, and MVI. Immunohistochemistry (IHC) for CD34 (mouse monoclonal Q-BEnd-10, Dako A/S, pre-Diluted Copenhagen Denmark) was automatically performed on FFPE 2-μm-thick sections with Benchmark ULTRA immunostainer (Ventana/Roche, Ventana Medical Systems). Nestin (mouse monoclonal 10C2, 1:400 dilution, Millipore USA) immunostaining was manually performed as previously described [21] with NovoLink Polymer Detection Kit (Novocastra, Newcastle, UK).

The four main patterns of HCC vascular modifications were determined according to the qualitative sinusoidal and arteriolar positivity for CD34 and Nestin, as well as tumor architecture, as previously described (4). Briefly, “Pattern a” was defined in HCCs with microtrabecular and acinar architecture, with CD34+/Nestin- sinusoids; “pattern b” in HCCs with similar architecture, but showing CD34+/Nestin+ sinusoids; “pattern c” was defined in HCCs with macrotrabeculae surrounded by a CD34+/Nestin+ endothelium; “pattern d” characterized solid HCCs with new-formed CD34+/Nestin+ arteries (Fig. 1) [5]. These vascular patterns were found only in HCCs, as they reflect neoplastic modifications of the vascularization, and they are not already occurred in healthy liver and/or in cirrhosis.

To address the important issue of the choice of tissue control for normalization in miRNA array, we selected two different pools:

1. A pool composed of 10 healthy livers obtained from healthy multiorgan donors (five males and five females; mean age 64.2 ± 10.2 years, range 48–75). This tissue was retrieved during the histological graft evaluation for donor safety in our Institution. Of note, this selected tissue is virus-free and cancer-free.

2. A pool composed of 10 cirrhotic livers obtained from practice routine analyses (five males and five females; mean age 55.2 ± 8.4 years, range 46–72), all affected by HCV-driven end-stage liver disease.

miRNA Arrays

RNA Extraction

A commercial kit was used for RNA extraction from FFPE sections (FFPE Recover All, Life Technologies, Carlsbad, CA, USA). Tissue slides were dewaxed with xylene. Tissue was recovered from the slides with a scalpel by adding 10 μl of digestion buffer. The protocol was followed as stated by manufacturer’s instruction. The RNA quality and concentration were evaluated by using a ND-1000 spectrophotometer (NanoDrop, Fisher Thermo, USA); RNA was considered pure if A260/A280 ratio = 1.9–2.1.

Megaplex Reverse Transcription Reaction

The reverse transcription assay was performed using the TaqMan® MicroRNA Reverse Transcription Kit associated with the Megaplex RT Primers Pool A and B (Life Technologies). Samples were loaded in the GeneAmp PCR System 9700 (Life Technologies) following the manufacturer’s thermal conditions.

cDNAs Megaplex Pre-amplification mix

To increase the yield of cDNAs, a second step of pre-amplification was performed with the TaqMan® PreAmp Master Mix associated with Megaplex PreAmp Primers Pool A and B (Life Technologies). Samples were loaded in the GeneAmp PCR System 9700 following the manufacturer’s thermal conditions. Finally, the pre-Amp products were diluted in 75 μL of H2O.

RT-PCR Arrays

Relative quantitation of targets was performed with TaqMan Human microRNA Arrays 384-wells (Gene expression Micro Fluidic card, Array A v2.1 e Array B v3.0, Life Technologies). PCR Reaction mix was composed by 450 μL of TaqMan Universal Master Mix II, no Amperase UNG (Life Technologies), 9 μL of diluted PreAmp product with primer Pool A or B. (prepare two separate reaction tubes) and 441 nuclease free water. Dispense 100 μL of the sample-specific PCR reaction mix (Pool A or B) in each fill port of the 384 plate in Array A or B. Amplification was performed on the Real Time 7900HT System (Life Technologies), and data were collected with
the SDS software v2.2 (Life Technologies). Cycling conditions were as follows: 2 min at 50 °C, 10 min at 94.5 °C, 30 s at 97 °C (40 cycles), 1 min at 59.7 °C. The transcription levels were normalized using RnU44 as a reference gene. The expression values for HCC are presented as fold expression in relation to controls livers; the actual values were calculated using the $2^{-\Delta\Delta C_T}$ equation, where $\Delta\Delta C_T = [\text{CT Target} - \text{CT Rnu44}]_{\text{target sample}} - [\text{CT Target} - \text{CT GUSB}]$ (control sample).

**Statistics and Data Analysis**

Variables were expressed as means ± standard deviations, ranges, and frequencies. MicroRNA Arrays expression was analyzed with ExpressionSuite Software v1.0 (Life Technologies). The hypothesis-testing procedure was applied once the $C_t$ values were normalized with the endogenous gene and the control groups. Student’s $t$ test was applied to compare the means of the normalized $C_t$ values between two groups. Resulting $P$ values were adjusted to control the type I error rate using the Benjamini–Hochberg method [22]. miRNAs with a significant differential expression compared to controls were plotted on a Volcano plot.

**Results**

**Histopathology and Vascular Patterns**

Mean tumor size of the 22 HCCs analyzed was cm 6.33 ± 4.74 (range cm 1.60–18.0); Edmondson’s grade was 2 in 1 (4.5%), 3 in 17 (77.3%) and 4 in 4 (18.2%) cases. HCCs showed infiltrative margins in 19 (86.4%), and MVI was observed in 17 (77.3%) cases. Twelve
Fig. 2 Significant differential miRNA expression in HCC in relation to the control group, healthy liver (red bars) or cirrhotic liver (blue bars). The X axis represents the log10 (fold change); down-regulated miRNAs are on the left part of the Y axis and up-regulated miRNAs are on the right part of the Y axis.
(54.5%) HCCs arose in a cirrhotic liver (c-HCC) and 10 (45.5%) in a non-cirrhotic livers (nc-HCC).

According to HCC architecture and IHC for CD34 and Nestin, the HCCs of our series were sorted in vascular “pattern a” in 2 (9.1%), “pattern b” in 6 (27.3%), “pattern c” in 8 (36.3%), and “pattern d” in 6 (27.3%) cases (Fig. 1) (4).

**miRNA Analysis I: Deregulated miRNAs in HCCs**

The first step of our study was to assess the miRNAs deregulated in HCC. After the Benjamini–Hochberg posttest, we found 14 miRNAs significantly deregulated compared to the healthy liver control pool (see Figs. 2, 3, Supplemental table S1). In particular, 5 miRNAs were down-regulated (mean fold increase 0.12 ± 0.07) and 9 up-regulated (mean fold increase 8.77 ± 7.42). Using the cirrhotic liver control pool, we noticed that 53 miRNAs were significantly deregulated. In particular, 44 were down-regulated (mean fold increase 0.10 ± 0.11), and 9 up-regulated (mean fold increase 103.55 ± 223.29).

Fifty-five miRNAs gave results depending on the tissue used for normalization: 8 miRNAs were limited to the analysis using the healthy liver control pool, while 47 were limited to the analysis using the cirrhotic liver control pool. Conversely, the deregulation of 6 miRNAs was common between the two analyses, 5 with the same trend (miR-34a, miR-93, miR532, miR-149#, and miR-7f-2#) and 1 with the opposite trend (miR-30a-5p) (Fig. 3). Among these 6 significant miRNAs, 5 are already known to be associated with HCC or with other human cancers (Table 1). No correlations emerged with etiology in our series (data not shown), except for miR-149# that showed a significant down-regulation in HCC with HCV- and HBV-driven chronic liver disease ($P < 0.001$). All raw data prior to statistical analysis are found in the Supplemental table S2 (sheet 1).

**miRNA Analysis II: Deregulated miRNAs in c-HCCs and nc-HCCs**

When the healthy liver control pool is used for normalization, 14 miRNAs were significantly down-regulated in
HCC tissue (Table 2, Supplemental table S2, sheet 2): particularly, only miR-1178 was significantly down-regulated in c-HCCs, while the remaining 13 miRNAs were deregulated in nc-HCCs. Among these 14 miRNAs, 5 were in common with those resulting in analysis I (all 22 HCCs): miR-149#, miR-23a#, miR-640, miR-1259, and miR-7f-2#.

Remarkable results were obtained using the cirrhotic liver control pool for normalization: 120 miRNAs were found to be deregulated (Table 2, Supplemental table S2, sheet 2), 37 in c-HCCs (all down-regulated but 2) and 83 in nc-HCCs (all down-regulated). Forty-three out of 120 miRNAs were common with those observed in general HCCs (analysis I). Furthermore, 29 down-regulated miRNAs were common to c-HCCs, nc-HCCs and the overall HCCs, in all cases with the same trend, confirming the results of analysis I. It is crucial to notice that the pool of cirrhotic control tissues might not be an appropriate model for the normalization of nc-HCCs.

Three miRNAs (miR-136#, miR-149# and 188-3p) were significantly down-regulated independently from the analyzed tissues (c-HCC or nc-HCC) and the pool used to compare the fold change (Supplemental table S2, sheet 2).

### miRNA Analysis III: Deregulated miRNAs in HCCs with Different Vascular Patterns

The analysis of the miRNAs expressed in HCCs with different vascular modifications showed that each of the four vascular patterns is characterized by the differential expression of miRNAs. In particular, compared to the healthy liver control pool, 16 miRNAs were significantly deregulated, 1 in “pattern a” HCCs, 4 in “pattern b,” 6 in “pattern c” and 5 in “pattern d” HCCs (Table 3, Supplemental table S2, sheet 3). Compared to the cirrhotic liver control pool, 112 miRNAs were significantly deregulated, 4 in “pattern a” HCCs (not the same as above), 16 in “pattern b,” 56 in “pattern c” and 36 in “pattern d” HCCs (Table 3, Supplemental table S2, sheet 3).

Interestingly, HCCs with patterns “a” and “b” share no miRNAs in common using the two different normalizations. Conversely, both HCCs with “pattern c” and HCCs with “pattern d” show 3 common miRNAs, all of them with similar fold changes, using healthy or cirrhotic liver control pools as reference. “Pattern a” HCCs are the only biological group showing up-regulation of 2 miRNAs (miR-551b# and miR-1825), while all other cases were down-regulated (all data are found in the Supplemental table S2, sheet 3). Patterns “a” and “b” HCCs do not conserve the same profile using different control tissues, while pattern “c” and “d” seem to show peculiar miRNA profiles (see “Discussion”).

### Discussion

The present study focused on a series of surgically resected histologically advanced HCCs, with the aim (1) to define the different miRNA profiles of HCCs arisen in cirrhotic and non-cirrhotic livers based on two different human liver controls extracted from healthy and cirrhotic livers; (2) to
correlate miRNA expression with the patterns of tumor vascular modifications according to the same two controls. The tissue used for normalization represents a key point in order to identify miRNAs deregulation in HCCs. Since HCCs can arise in both cirrhotic and non-cirrhotic livers (albeit more frequently in the former), we carried out all analyses using RNA extracted from both tissues, in the present study. Total RNA extracted from cirrhotic liver cannot be considered an appropriate control for all cases, in particular for nc-HCCs. Even if nc-HCCs usually arise in a context of chronic hepatitis, we decided here to utilize healthy multiorgan donors without virus exposure, chronic liver disease or neoplasia. Most recent papers on miRNAs have studied both HCC and cirrhosis using healthy liver tissue as Refs. [6, 13, 14], with weak overlaps on the identified HCC-related miRNAs, and often discordance between the up- and down-regulation of specific miRNAs. This last issue is very important, since it was postulated that the up- or down-regulation of miRNAs could determine the prognosis in HCC patients [17] and could be used in specific inhibition or restoration therapies [23]. Beside the factors that can influence the final results of miRNA analysis, such as the choice of the array used, the choice of the housekeeping genes, and the statistical approach [24], the selection of the reference control is a key step that can impact the expression and the fold

<table>
<thead>
<tr>
<th>Biological groups</th>
<th>Control pool</th>
<th>Normal</th>
<th>Cirrhotic</th>
<th>Common miRNAs according to normalization pools</th>
</tr>
</thead>
<tbody>
<tr>
<td>nc-HCC</td>
<td>miR-149#, miR-188-3p, miR159a, miR-543, miR-483-3p, miR-640, miR-1259, miR-23a#, 7f-2#, miR-1243, miR-136#, miR-562</td>
<td>miR-30a-3p, miR-1274B, miR-769-3p, miR-617, miR-720, miR-213, miR-30a-5p, miR-26a-2#, miR-1260, miR-132#, miR-29b-2#, miR-543, miR-429, miR-331-5p, miR-635, miR-200c, miR-542-5p, miR-186#, miR-136, miR-20a#, miR-571, miR-181e#, miR-24-2#, miR-223#, miR-376a, miR-99a#, miR-125b-2#, 7f-2#, miR-144#, miR-590-3P, miR-596, miR-886-5p, miR-26a-1#, miR-145#, 7a#, miR-650, miR-380-5p, miR-149#, miR-31#, miR-338-3p, miR-199b, miR-199a, miR-1208, miR-1288, miR-214#, miR-126#, miR-483-3p, miR-483-5p, miR-200a, miR-662, miR-497, miR-1290, miR-200b, miR-141, miR-644, miR-1247, miR-130a#, miR-136#, miR-200a#, miR-584, miR-195#, miR-656, miR-486-3p, miR-604, miR-646, miR-1255A, miR-380-3p, miR-519a, miR-609, miR-889, miR-378, miR-639, miR-1305, miR-188-3p, miR-202#, miR-30c-1#, miR-377#, miR-452#, miR-520b, miR-520D-3P, miR-551a, miR-621, miR-3875-5p</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>c-HCC</td>
<td>miR-1178</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Common miRNAs</td>
<td>None</td>
<td></td>
<td></td>
<td>miR-136#, miR-149#, miR-188-3p, miR-7a#, miR-125b-2#, miR-126#, miR-1305, miR-132#, miR-145#, miR-200a miR-200a# miR-202# miR-20a# miR-214# miR-26a-1# miR-30c-1#, miR-31#, miR-338-3p, miR-378, miR-380-5p, miR-452#, miR-520D-3P, miR-551a, miR-590-3p, miR-656</td>
</tr>
<tr>
<td>between nc-HCC</td>
<td></td>
<td></td>
<td></td>
<td>miR-136#, miR-149#, miR-188-3p, miR-543</td>
</tr>
</tbody>
</table>
increase in miRNAs. The same concept was already expressed by Visani et al. [19] in brain tumors.

According to our data, when we used the cirrhotic liver control pool as reference, a greater number of miRNAs was significantly deregulated in each analysis among biological groups. Moreover, even the expression of an endogenous housekeeping gene (U6 snRNA) resulted heterogeneous in one analysis (Table 3): it could be hypothesized that cirrhosis is a too heterogeneous model to be used as control, albeit representing the background of most HCCs, due to a high percentage—and variability—of inflammatory cells, ductular reaction, fibrosis, etc. [17]. For the same reason, we found many differences in miRNAs deregulation between c-HCC and nc-HCC (Table 2).

At any chance, our results showed 6 miRNAs to be deregulated in HCCs, despite the control used: miR-532, miR-34a, miR-93, miR-149#, miR-7f-2#, and miR-30a-5p. Apart from miR-532, all of these miRNAs have been already reported in the HCC literature (Table 1).

miR-34a plays a tumor suppressor role through the regulation of c-Met and β-catenin pathways [25, 26]. miR-34a deregulation was linked to HCC progression and aggressiveness both in vivo and in vitro. Interestingly, a very recent paper correlated the deregulation of miR-34a with the recurrence rates of early HCC after radiofrequency [27].

A deregulation of miR-93 significantly correlated with poor prognosis in HCCs. In vitro miR-93 activity was also correlated with HCC sensitivity to sorafenib and tivantinib [18]: the authors concluded that miR-93 was involved in cell proliferation, migration, and invasion through the oncogenic c-Met/PI3 K/Akt pathway, and it also inhibited apoptosis by PTEN and CDKN1A pathways [18]. Thus, the use of synthetic regulators of miR-93 may prove to be a promising approach to liver cancer treatment [28]. Other studies have found a deregulation of miR-93, together with other miRNAs, during HCC progression [29, 30].

miR-149 modulates the Akt/mTOR pathway in HCC. A tumor suppressive role for miR-149, and a prognostic role in human HCCs were hypothesized [7].

miRNA let-7f-2 was found to be down-regulated in HCCs [31, 32]. It was seen that the let-7f family of miRNAs potentiates sorafenib-induced apoptosis in human HCC [16]. Recently, a study correlated also serum let-7f levels with HCC size and early recurrence [33].

Finally, miR-30a-5p expression was altered in HCC compared to adjacent tissue [34]. In another study, the overexpression of miR-30a-5p inhibited cell proliferation, induced apoptosis, increased the number of cells in S phase, and markedly inhibited invasion and migration of HCC cells in vitro and in vivo [35].

### Table 3 miRNA deregulated in each pattern of vascular modification in HCC compared to the normal or cirrhotic controls

<table>
<thead>
<tr>
<th>Biological groups</th>
<th>Normal</th>
<th>Cirrhotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pattern a</td>
<td>miR-511b#</td>
<td>miR-922, miR-548d-5p, miR-516-3p, miR-1825</td>
</tr>
<tr>
<td>Pattern b</td>
<td>miR-149#<em>, miR-1178</em>, miR-1243, miR-23a#</td>
<td>U6 snRNA, miR-889, miR-770-5p, miR-662, miR-616, miR-520b, miR-519b-3p, miR-33a, miR-335#, miR-31#, miR-29a#, miR-200a#, miR-19b-1#, miR-195#, miR-125b-2#, miR-101#</td>
</tr>
<tr>
<td>Pattern c</td>
<td>miR-149#<em>, miR-1178</em>, let-7a#, miR-1300, miR-483-3p, let-7 g#</td>
<td>miR-29#, miR-99a#, miR-943, miR-92b#, miR-758, miR-720, miR-708, miR-688, miR-665, miR-661, miR-644, miR-628-3p, miR-609, miR-604, miR-601, miR-596, miR-592, miR-590-3p, miR-572, miR-571, miR-497, miR-483-3p, miR-429, miR-33a#, miR-338-3p, miR-30e-3p, miR-30a-5p, miR-30a-3p, miR-29b-2#, miR-26a-1#, miR-218, miR-214#, miR-20a#, miR-202#, miR-200c, miR-188-3p, miR-186#, miR-16-1#, miR-149#, miR-145#, miR-144#, miR-141, miR-132#, miR-1303, miR-1290, miR-1288, miR-1267, miR-1260, miR-1260, miR-125b-1#, miR-1228#, miR-1208, miR-1201, let-7i#, let-7a#</td>
</tr>
<tr>
<td>Pattern d</td>
<td>miR159a, miR-541#, miR-875-5p, miR-149#*, miR-675</td>
<td>miR-491, miR-875-5p, miR-769-3p, miR-675, miR-656, miR-650, miR-646, miR-639, miR-621, miR-584, miR-567, miR-551a, miR-548L, miR-548b, miR-541#, miR-520D-3P, miR-512-5p, miR-452#, miR-378, miR-377#, miR-30c-14-, miR-223#, miR-200b, miR-200a, miR-190b, miR-198, miR-143#, miR-140-3p, miR-136#, miR-130a#, miR-1305, miR-1274, miR-1274A, miR-1255A, miR-1247, miR-1225-3P</td>
</tr>
</tbody>
</table>

The same concept was already expressed by Visani et al. [19] in brain tumors.

According to our data, when we used the cirrhotic liver control pool as reference, a greater number of miRNAs was significantly deregulated in each analysis among biological groups. Moreover, even the expression of an endogenous housekeeping gene (U6 snRNA) resulted heterogeneous in one analysis (Table 3): it could be hypothesized that cirrhosis is a too heterogeneous model to be used as control, albeit representing the background of most HCCs, due to a high percentage—and variability—of inflammatory cells, ductular reaction, fibrosis, etc. [17]. For the same reason, we found many differences in miRNAs deregulation between c-HCC and nc-HCC (Table 2).

At any chance, our results showed 6 miRNAs to be deregulated in HCCs, despite the control used: miR-532, miR-34a, miR-93, miR-149#, miR-7f-2#, and miR-30a-5p. Apart from miR-532, all of these miRNAs have been already reported in the HCC literature (Table 1).

miR-34a plays a tumor suppressor role through the regulation of c-Met and β-catenin pathways [25, 26]. miR-34a deregulation was linked to HCC progression and aggressiveness both in vivo and in vitro. Interestingly, a very recent paper correlated the deregulation of miR-34a with the recurrence rates of early HCC after radiofrequency [27].
Conflict of interest

Compliance with ethical standards

Funding This work has been supported by the Programma di Ricerca Regione-Università 2010–2012, Regione Emilia-Romagna, bando “Ricerca Innovativa” (Professor L. Bolondi), project title “Innovative approaches to the diagnosis and pharmacogenetic-based therapies of primary hepatic tumors, peripheral B and T-cell lymphomas and lymphoblastic leukaemias.” The funding body had no role in the study design, in the collection, analysis, interpretation of data, in the decision to submit the manuscript and in the decision to publish.

References


