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A large set of miRNAs is dysregulated since the earliest steps of human hepatocellular carcinoma development

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ABSTRACT

Hepatocellular carcinoma (HCC) mostly results from a stepwise process characterized by the development of premalignant lesions, such as low- or high-grade dysplastic nodules (LGDN and HGDN, respectively) in a cirrhotic setting. MicroRNAs (miRNAs) are small noncoding RNAs involved in post-transcriptional regulation of gene expression that can act as oncogenes or tumor suppressors. Whether and which miRNAs are involved in the early stages of HCC development remains elusive. Here, small RNA sequencing was applied to profile miRNA expression in 55 samples (cirrhotic nodules, CNs), LGDNs, HGDNs, early HCCs, and small progressed HCCs, obtained from 17 patients bearing HCCs of different etiology. A miRNA expression signature of 62 miRNAs distinguishing small progressed HCCs from matched CNs was identified. Interestingly, 52 of these miRNAs discriminated CNs from LGDNs/HGDNs, regardless of the etiology, and remained modified along the tumorigenic process. Functional analysis of the predicted mRNA targets of deregulated miRNAs identified common modifications between early and late stages of HCC development likely involved in the stepwise process of HCC development. Our results demonstrate that miRNA deregulation happens very early in human liver carcinogenesis, implying their critical role in the tumorigenic process. The identification of miRNAs discriminating cirrhotic from neoplastic nodules may have relevant translational implications for early diagnosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second cause of cancer-related deaths worldwide and a major health problem. Multiple viruses, chronic metabolic alterations leading to chronic inflammation, and epigenetic and genetic changes cooperate in cancer development *via* a combination of common and distinct etiology-specific pathways. Genome-wide gene expression microarray and quantitative real-time reverse transcriptase PCR studies have tried to identify the genes abnormally expressed in HCC and to generate molecular signatures able to distinguish among different types of liver tumors and between tumors with different outcome [1,2]. These studies indicated a general aberrant activation of signaling pathways involved in cellular proliferation, survival, differentiation, and angiogenesis, which are heterogeneously present in each tumor. However, they did not lead to a consensus for a signature or for a single pathway that is prominent and characteristic of HCC.

Recently, it has been suggested that the classification and stratification of tumors can be performed not only on the bases of mRNA expression analysis, but also—and likely more accurately—on the modulation of microRNAs (miRNAs), small noncoding RNAs that negatively control gene expression [3]. miRNA involvement in cancer development is now well-established and miRNA expression profiles accurately classify tumors at different stages and distinguish among subsets of patients with different molecular alterations [4]. Changes in the expression of miRNAs between tumor specimens and their corresponding peritumoral tissues have been investigated also in HCC but mostly in advanced tumors [2]; however, very little is known on the earliest phases of the tumorigenic process [5]. Moreover, results were often discordant and without a clear identification of miRNAs critical for HCC development and progression [6,7]. Indeed, microarray profiling of mRNAs and miRNAs performed on human advanced HCCs does not help to discriminate the

alterations which are driving the tumorigenic process from those which are the consequences of full malignant transformation. Unfortunately, knowledge of the molecular events in early stages of human HCC development is hampered by the clinical difficulty to diagnose and study early lesions. Notably, experimental studies have shown that alteration of miRNA expression is an early event in hepatocarcinogenesis and that a panel of 13 miRNAs is dysregulated throughout the carcinogenic process [8]. This finding suggests that the analysis of the expression of miRNAs at early stages of hepatocarcinogenesis may provide relevant information on the miRNAs driving hepatic tumorigenesis.

To investigate whether miRNAs are dysregulated since the early stages of hepatocarcinogenesis also in humans, we applied small RNA sequencing to search for liver miRNAs and to profile their expression patterns in CNs, LGDNs, HGDNs, eHCCs, and pHCCs, analyzing 55 samples from 17 patients mostly with HCV-related HCC, aiming at identifying possible relationships between these miRNAs and HCC onset and progression. A validation study on a handful of selected miRNAs of samples obtained from 12 additional patients was also performed to further confirm and extend our investigation.

MATERIALS AND METHODS

Sample collection

Resected specimens from 17 patients, each with multiple hepatocellular nodules (HN) well representative of different steps of human hepatocarcinogenesis, were included in this study. After proper identification of the hepatocellular nodule on the H&E section, the lesion was manually microdissected from sequential, matched 10 μm paraffin-embedded sections. These tissue samples harbored 61 HN (mean: 3.5 HN/patient; range 2 to 6 patient) as follows: 17 cirrhotic nodules (CNs), nine LGDNs, six HGDNs, six eHCCs, and 23 pHCCs. Nodules were histologically characterized according to criteria outlined by the

International Consensus Group on HCC nomenclature [9]. All cases of pHCC had a maximum diameter not greater than 2 cm. Stromal invasion was a feature to identify eHCC and distinguish it from HGDNs, whereas pHCC showed an overt infiltrative growth. Clinical and pathological features of the series are illustrated in **Supplemental Table S1**. Of these 61 nodules, 55 had sufficient material for a complete morphological characterization and molecular analysis, namely 14 CNs, nine LGDNs, six HGDNs, six eHCCs, and 20 pHCCs. To further support the finding that dysregulation of miRNAs is a very early event in HCC development, RT-qPCR was performed in an additional series of LGDNs, HGDNs, eHCCs, and pHCCs from 12 additional patients with mostly HCV-related HCC (**Supplemental Table S2**). All patients have given their informed consent and this study was conducted according to guidelines and regulations by the Research Ethics Committee of the Humanitas Research Hospital, as explicated by formal approval to M.R. of projects regulating the use of retrospective solid tumor tissues (Approval Code obtained by Ethic Committee is n.13/17, 15/6/2017).

RNA purification and small RNA sequencing

Total RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) sections of human livers using miRNeasy FFPE Kit (QIAGEN GmbH, Hilden, Germany) in duplicates and quantitated with NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Cinisello Balsamo, Italy). To minimize miRNA degradation in archival miRNA studies [10], only freshly-cut sections from paraffin blocks stored for less than 12 years were used.

For smallRNA-seq, 1µg of total RNA/sample was used for library preparation with Illumina TruSeq smallRNA sample preparation Kit and each library (8pM) was sequenced on HiSeq2500 (Illumina) for 50 cycles at Genomix4life (Genomix4life S.r.l., c/o Laboratory of Molecular and Genomic Medicine, Baronissi, Italy). Raw smallRNA-Seq data are available in ArrayExpress database (https://www.ebi.ac.uk/arrayexpress/; accession: E-

MTAB-3973) [11]

Bioinformatics analysis

The sequencing reads from each sample were processed using iSmaRT [12] to detect human miRNAs annotated in miRBase (v21). The expression values of miRNAs were represented as read per million (RPM) values making them comparable across samples and to filter out low expression molecules *filtered data* function of R package (BRB-Array Tools, *P*-value of the log-ratio variation in greater than 0.05, 50th percentile of expression values <10) was used.

Differential expression analysis between different tissues were performed with R using F-test (with Random-Variance Model) and Multivariate Permutations test. miRNAs were considered differentially expressed when showing absolute Fold-Change (FC) ≥2.

RT-qPCR

miRNAs were extracted from FFPE sections of human livers using miRNeasy FFPE Kit (QIAGEN GmbH, Hilden, Germany) and quantitated with NanoDrop-1000 spectrophotometer. miRNAs were retro-transcribed starting from 0.4µg RNA/sample using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Analysis of let-7a-5p, miR-375, miR-21-5p, miR-200a-3p, miR-200b-3p, miR-429, miR-141-3p, miR-16-5p, miR-151b, and miR-224-5p expression was performed using specific TaqMan probes (Applied Biosystems) and RNU48 as endogenous control. Relative quantification analysis for each miRNA was calculated by the $2^{-\Delta\Delta Ct}$ method. One-way analysis of variance (ANOVA was used to analyze the data (Instat; GraphPad, San Diego, CA). The results of observations are presented as the means ± SE. A value of $P < 0.05$ was regarded as a significant difference between groups.

Functional analysis by means of the Ingenuity IPA Software

Human standard miRNA symbol were submitted to the Ingenuity Pathway Analysis (IPA) pipeline. Analysis of the function was based on the number of miRNA significantly dysregulated (fold difference cutoff + 2.0) with corresponding biological functions, with the restriction of at least 14 miRNAs per function to emphasize the functions with most miRNAs differentially expressed. The significance of each network and the connectivity was estimated in IPA.

RESULTS

The miRNA expression pattern discriminates cirrhotic from neoplastic lesions

To identify miRNAs dysregulated along human hepatocarcinogenesis, small RNA-Seq was performed on RNAs extracted from cirrhotic nodules (CNs=14), LGDNs (n=9), HGDNs (n=6), eHCCs (n=6), and pHCC (n = 20) obtained from 17 patients (**Supplemental Table S1**). After normalization in RPM, 2,064 miRNAs were selected. Following appropriate filtering on all hepatic lesions (*Materials and Methods*), 154 miRNAs presented reproducible up- or down-regulation in neoplastic lesions with respect to peritumoral tissue (**Supplemental Table S2**). Unsupervised hierarchical clustering analysis revealed two major clusters; however, although neoplastic lesions largely grouped in one cluster, no clear stratification of the different lesions was evident (**Supplemental Fig. S1**).

The analysis was thus restricted only to samples from patients carrying HCV-related HCC, the most frequent etiologic agent in our series of cases. Under this condition, 126 miRNAs were differentially expressed in the two sets of samples. Hierarchical clustering revealed a good segregation of neoplastic from cirrhotic lesions in two separated clusters. Indeed, one cluster included neoplastic lesions (LGDNs, HGDNs, eHCCs, and pHCCs) and only 1/6 CNs, and the other comprising all remaining CNs and only 3/22 neoplastic lesions (**Fig.1).**

A set of miRNAs is dysregulated in small progressed HCCs

To investigate miRNAs differentially expressed in pHCCs from HCV patients as compared to matched cirrhotic tissues, Random-Variance Model (F-test) and the Multivariate Permutation Test (Fold Change, |FC| ≥ 2.0) were applied. Following this analysis, 36 miRNAs were found up-regulated and 34 down-regulated (**Table 1**) in pHCC samples as compared to their peritumoral cirrhotic tissues. Interestingly, out of these 70 dysregulated miRNAs, 62 were differentially expressed as compared to cirrhotic nodules also in HCV-unrelated pHCCs (**Table 1).** This result suggests their relevance in HCC progression regardless the viral etiology of the chronic liver disease.

miRNAs are already deregulated in the earliest stages of hepatocarcinogenesis

Human hepatocarcinogenesis is a multistep process mostly arising in a chronically inflamed and remodeled hepatic microenvironment and proceeding from not malignant neoplastic lesions such as dysplastic nodules (low and high grade) to early malignant tumors (eHCC) and, ultimately, to small but yet progressed HCC (pHCC) [13]. Therefore, it was investigated if and which miRNAs are differentially expressed since the earliest stages of tumorigenesis. Surprisingly, of the 62 miRNAs dysregulated in pHCCs, 52 were already altered in HCC precursors (**Table 2**), suggesting their involvement in HCC onset. Indeed, 28 miRNAs showed a low expression in CNs that significantly increased in LGDNs, remaining high across all the spectrum of pathological stages analyzed, up to pHCCs. In contrast, 24 miRNAs displayed an opposite behavior, as their expression was significantly higher in the cirrhotic tissue; however, it became very low or even absent throughout the tumorigenic process.

Overall, these results demonstrate that a panel of 52 miRNAs can discriminate between CNs and early neoplastic lesions.

To further support that miRNAs dysregulation is a very early event in HCC development, RT-qPCR was performed in a validation series of LGDNs (4), HGDNs (3), eHCCs (4), and pHCCs (3) from 12 HCC patients with different etiology, whose clinical data are shown in (**Supplemental Table S3)**. For this analysis nine miRNAs (miR-429, miR-141-3p, miR-200a-3p and miR-200b-3p, miR-375, miR-21-5p, miR-16-5p, miR-224-5p, miR-151b) identified in the discovery setting and known to be dysregulated in advanced human HCC, were selected [6,14]. The results observed by NGS in the first set of samples was confirmed for all six miRNAs, that were profoundly dysregulated since the dysplastic nodule stage, as compared to matched cirrhotic parenchyma; interestingly, the vast majority of them were down-regulated in the neoplastic lesions, with the only exception of miR-21-5p, whose expression was strongly up-regulated at all stages of the process (**Fig.2**). These results further support the concept that alterations of several miRNAs occur before the setting of biological and morphological features that characterize cancer cells (nuclear and architectural atypias, invasiveness, vascular changes).

Another interesting observation stemming from NGS analyses is that let-7a-5p showed a progressive and highly significant decrease from dysplastic nodules to early and progressed HCCs (**Fig.3**), suggesting its possible involvement in the transition to an overt malignant condition. Accordingly, a similar trend, although not reaching statistical significance, was observed by RT-qPCR analysis, in a validation setting.

Ingenuity Pathway Analysis in dysplastic nodules and HCC

Ingenuity Pathway Analysis (IPA) of miRNAs revealed that most of the dysregulated miRNAs are commonly modified in neoplastic nodules and in pHCCs and are involved in pathways associated with cancer and hematological disease **(Supplemental Fig. S2).** Analysis of networks differentially activated in neoplastic nodules and in pHCC revealed molecular relationships between TP53 and dysregulated miRNAs. In fact, of the 52 miRNA

found dysregulated in neoplastic nodules (from LGDNs and HGDNs), 10 showed direct relationship with TP53 node in both dysplastic nodules and pHCCs **(Fig.4)**

DISCUSSION

Investigations on HCC precursors and early malignant lesions may help to clarify the molecular mechanisms of hepatocarcinogenesis, most of which still remain obscure. Moreover, their identification may have important clinical significance for the potential translational implications in terms of tumor prevention, early diagnosis, and personalized treatment.

Among the possible genetic/epigenetic changes taking place during hepatocarcinogenesis, miRNA dysregulation is important both in pathogenesis and in progression of human HCC. However, the vast majorities of the studies have been so far finalized to validate these molecules (individually or as expression signature) in patients at an advanced stage of the disease [2].

Here, miRNA deregulation was studied in a very selected setting of lesions encompassing the full spectrum of early human hepatic carcinogenesis, consisting of a variety of hepatocellular nodules ranging from dysplasia (LGDN, HGDN) to early and progressed (but not advanced) small HCCs (up to 2 cm in size). miRNA changes were evaluated and compared to those of the adjacent liver (chronic liver disease/cirrhosis), in a cohort of patients mostly harboring HCV-related HCC.

The most significant finding stemming from this work consists in the identification of a large set of miRNAs consistently deregulated since the earliest steps of tumorigenesis. Indeed, analysis of the expression levels of these molecules by small RNA sequencing allowed discriminating, in the HCV-related cohort, the hepatitis/cirrhotic background from malignant hepatocellular nodules, with a set of 70 miRNA discriminating pHCC from the adjacent liver. Notably, 62 of these could also clearly discriminate pHCCs from the peritumoral liver

tissue independently of the etiology (viral, non viral), with 52 out of these (90%) showing consistent alterations already in non-malignant neoplastic lesions (LGDNs and HGDNs), which persisted to variable degrees up to eHCCs and pHCCs.

The similarities in miRNA dysregulation between dysplastic nodules and early and progressed HCCs is also confirmed by IPA analysis based on the known functions encoded by their target mRNAs, which showed involvement of the same functional categories (ie, cancer, immunological disease, gastrointestinal disease) and the same network, with TP53 as the main node.

Notably, in a different clinical setting (HBV-related background) consisting of lesions quite comparable for number, type, and size to those included in this study, comparable results were reported. [5]. Taken together, these findings demonstrate that miRNA changes are among the earliest molecular hallmarks of hepatocarcinogenesis, regardless of the specific etiology.

This finding is of particular interest as Nault et al [15] have shown that genetic changes rarely occur before HCC onset, TERT mutation being the most notable exception. Moreover, Marquandt et al [16] demonstrated relatively modest transcriptomic changes in precursor lesions preceding HCC appearance; on the opposite, epigenetic changes have been consistently reported in HCC precursors [16-18].

Overall, these results suggest that inhibition of specific genes/pathways by DNA hypermethylation and miRNA deregulation tend to accumulate in multistep hepatocarcinogenesis, paving the way to HCC development.

To further confirm and validate the results obtained in the first set of analysis, a panel of nine miRNAs known to be dysregulated in human HCC (miR-21-5p, miR-375, miR-200a-3p, miR-200b-3p, miR-141-3p, miR-429, miR-16-5p, miR-224-5p, and miR-151b) was selected for RT-qPCR analysis in an additional set of hepatocellular dysplastic and malignant lesions **(Fig. 2).** The results obtained not only confirmed dysregulation of these

miRNAs in pHCCs, but also the same trend of up- or down-regulation shown by NGS analysis along the tumorigenic process, as compared to the adjacent hepatitis/cirrhosis. These data further suggest a critical role of miRNAs in the shift from a pure non-tumoral inflammatory/fibrotic setting (hepatitis/cirrhosis) to premalignant and malignant lesions (hepatocellular dysplastic and malignant nodules). miRNA modulation in hepatocarcinogenesis is likely to undergo further changes during HCC progression, as shown by the emergence of C19MC cluster up-regulation in advanced HCC with vascular invasion [19].

Among early dysregulated miRNAs, miR-375 is of particular interest, since it has been shown to target YAP-1 (20,21], a transcriptional co-activator involved in the HIPPO pathway [22,23]. YAP-1 overexpression has been implicated in human, mouse, and rat hepatic tumorigenesis concomitantly with down-regulation of miR-375 [24-28]. Accordingly, the present study shows an early down-regulation of miR-375 from LGDN up to small HCC, supporting a critical tumor suppressor role of this miRNA. Notably, Zhou et al [29] have shown that miR-375 down-regulation in HCC is significantly correlated with patient outcome, suggesting its dosage as potential prognostic biomarker, and Xue et al [30] recently proposed restoring miR-375 through gold nanoparticles delivery as a therapeutic approach in HCC.

Expression of Let-7 miRNA family is usually lost, reduced, or deregulated in most human cancers [31], and its repression in a HCC subset has been shown to be mediated by LIN28 overexpression [32]. Data show that expression of a member of this family, Let-7a-5p, progressively increases in dysplastic nodules, and it is followed by a 2-fold decrease in early and progressed HCC (**Fig. 3**), suggesting an involvement of this miRNA in the switch from dysplasia to cancer.

Furthermore, the miRNA 200 family (miR-200a, miR-200b, miR-141, miR-429) showed strong down-regulation in dysplastic nodules and HCCs compared to matched cirrhotic

tissues, in keeping with the finding that miR-200 family is up-regulated in liver fibrosis [33]. In many tumor types, including HCC, loss of expression of these miRNAs promotes epithelial-to-mesenchymal transition and metastasis [34, 35], and these molecules are also involved in malignant transformation and cancer development [36]. The results presented not only confirm a profound down-regulation of the miR-200 family in progressed HCCs, but also demonstrate that this down-regulation is already present in dysplastic nodules and eHCC, where overtly invasive properties and metastatic capabilities are not yet acquired.

Finally, miR-21 is up-regulated not only in HCC but also in precursor lesions. It has been previously reported that miR-21 is up-regulated in HCC tissues and hepatoma cell lines [29,37], and that its expression gradually increases along with an augmented invasive phenotype of the tumor. Moreover, miR-21 has been reported to play a role in hepatocyte proliferation by promoting cyclin D1 translation and inhibition of tumor suppressor (such as PTEN), and apoptotic (programmed cell death 4 (PDCD4) and B-cell lymphoma 2 (Bcl-2)) genes [38]. Even in this case, thus, the results obtained here are in line with the known role of miR-21 in promoting HCC.

In conclusion, the present work shows that HCC development is linked to profound alterations in the expression of a conspicuous set of miRNAs which occurs very early in the tumorigenic process. Demonstration that 52 miRNAs are deregulated along the sequence cirrhosis-HCC, suggests these molecules as critical players in HCC setting. These results consolidate our knowledge on the involvement of miRNAs and their targets in hepatocarcinogenesis, providing opportunities for the control of different and crucial signaling pathways of the tumorigenic process. Moreover, they may have relevant translational implications for early diagnosis and treatment. Interestingly, dysregulation of PIWI-interacting RNAs (piRNAs), another family of small nonconding RNAs that control transposon expression and the cell epigenome, has been recently reported to occur during

human liver carcinogenesis, from early stages to pHCC [11], further suggesting involvement of small regulatory RNAs in malignant transformation of liver cells.

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FIGURE LEGENDS

Figure 1. Hierarchical clustering of miRNA expression profile in cirrhotic nodules(CN), low-grade dysplastic nodules (LGDN), high-grade dysplastic nodules (HGDN), early hepatocellular carcinomas (eHCCs), and progressed HCCs (pHCCs) from HCV patients. Different types of lesions are indicated by colored bars. Red and green colors represent higher or lower expression levels of the miRNA (median centered), respectively.

Figure 2. RT-qPCR analysis of miR-200a-3p, miR-200b-3p, miR-429, miR-141-3p, miR-375, miR-21-5p, miR-16-5p, miR-224-5p, and miR-151b in cirrhotic nodules (CN), low-grade dysplastic nodules (LGDN), high-grade dysplastic nodules (HGDN), early hepatocellular carcinomas (eHCCs), and progressed HCCs (pHCCs). Relative quantification analysis for each miRNA was calculated by the $2^{-\Delta\Delta Ct}$ method. The histogram represents mean values ± SE of three to four samples per group; **P* < 0.05; **P < 0.005 compared to CNs.

Figure 3. RT-qPCR validation of miR Let 7a-5p in the stepwise development of hepatocellular carcinomas (HCC). miRNA expression was determined in cirrhotic nodules (CN), low-grade dysplastic nodules (LGDN), high-grade dysplastic nodules (HGDN), early HCCs (eHCCs), and progressed HCCs (pHCCs). MiRNA expression is reported as fold-change relative to cirrhotic nodules. **P* < 0.05 compared to eHCC and pHCCs.

Figure 4. Activated network in early (low-grade dysplastic nodules (LGDN)- high-grade dysplastic nodules (HGDN)) and late (progressed hepatocellular carcinomas (pHCC)) steps of hepatocarcinogenesis. Differentially activated network in LGDN-HGDN **(A)** and progressed HCC **(B)** showing TP53 as the main node. Red: up-regulated miRNA; green: down-regulated miRNA.

Table 1. MiRNAs differentially expressed between HCV-related pHCC compared to CN.

UniqueID	CIRR	pHCC	FC pHCC/CIRR
Up-regulated miRNAs			
hsa-miR-664a-3p	9.03	177.48	19.65
hsa-miR-194-5p	217.6	4177.61	19.20
hsa-miR-574-3p	14.34	258.14	18.00
hsa-miR-30b-5p	157.8	2235.23	14.16
hsa-miR-182-5p	182.02	2445.92	13.44
hsa-miR-148a-3p	3380.01	45111.2	13.35
hsa-miR-532-3p	2.24	29.14	13.01
hsa-miR-122-3p	95.74	1171.82	12.24
hsa-miR-126-5p	541.31	6328.81	11.69
hsa-miR-16-5p	490.81	5376.02	10.95
hsa-miR-26b-5p	755.48	7899.58	10.46
hsa-miR-197-3p	18.48	189.87	10.27
hsa-miR-15a-5p	67	681.34	10.17
hsa-miR-32-5p	19.96	199.19	9.98
hsa-miR-21-5p	6004.4	56869.68	9.47
hsa-miR-148b-3p	87.54	767.53	8.77
hsa-miR-26a-5p	3044.55	25508.39	8.38
hsa-miR-30c-5p	206.2	1685.54	8.17
hsa-miR-660-5p	52.67	427.34	8.11
hsa-miR-23b-3p	175.37	1391.91	7.94
hsa-miR-484	48.09	371.66	7.73
hsa-miR-24-3p	125.33	932.61	7.44
hsa-let-7f-5p	1312.47	9728.21	7.41
hsa-miR-103a-3p	621.93	3967.26	6.38
hsa-miR-224-5p	36.07	223.95	6.21
hsa-miR-151b	257.53	1561.55	6.06
hsa-miR-151a-5p	259	1564.01	6.04
hsa-let-7g-5p	698.84	3676.97	5.26
hsa-miR-10b-5p	2981.2	15335.95	5.14
hsa-miR-4284	13.97	71.1	5.09
hsa-let-7a-5p	1475.85	7509.3	5.09
hsa-miR-28-5p	78.38	389.6	4.97
hsa-miR-101-3p	1660.08	8219.51	4.95
hsa-miR-424-5p	89	437.34	4.91
hsa-miR-199a-3p=hsa-miR-199b-3p	646.23	3110	4.81
hsa-miR-27b-3p	7334.8	33296.42	4.54

Down-regulated miRNAs			
hsa-miR-483-5p	1459.15	24.66	0.02
hsa-miR-1275	10031.47	173.34	0.02
hsa-miR-874-3p	1561.12	61.02	0.04
hsa-miR-320b	4334.96	179.72	0.04
hsa-miR-320a	37905.85	1795.05	0.05
hsa-miR-3653-3p	1378.82	74.47	0.05
hsa-miR-138-5p	170.22	9.31	0.05
hsa-miR-4443	553.42	31.45	0.06
hsa-miR-200c-3p	132.71	8.49	0.06
hsa-miR-320e	242.83	15.64	0.06
hsa-miR-375	6578.63	434.33	0.07
hsa-miR-320c	475.1	40.29	0.08
hsa-miR-320d	390.16	33.18	0.09
hsa-miR-1291	402.24	37.57	0.09
hsa-miR-769-5p	7721.65	817.5	0.11
hsa-miR-30c-2-3p	464.1	57.22	0.12
hsa-miR-127-3p	18801.55	2732.21	0.15
hsa-miR-486-5p	18321.23	2689.5	0.15
hsa-miR-1246	7273.51	1070.49	0.15
hsa-miR-145-3p	1068.89	157.99	0.15
hsa-miR-1249-3p	100.64	16.24	0.16
hsa-miR-30c-1-3p	160.92	27.22	0.17
hsa-miR-146a-5p	8051.03	1382.08	0.17
hsa-miR-409-3p	1372.22	240.42	0.18
hsa-miR-29a-5p	187.78	33.68	0.18
hsa-miR-342-5p	71.49	13.21	0.18
hsa-miR-193a-5p	340.26	64.7	0.19
hsa-miR-382-5p	93.93	18.02	0.19
hsa-miR-193b-5p	139.56	27.68	0.20
hsa-miR-181c-3p	79.56	16.46	0.21
hsa-miR-1468-5p	98.68	23.61	0.24
hsa-miR-501-5p	81.03	19.8	0.24
hsa-miR-330-5p	74.34	21.06	0.28
hsa-miR-339-5p	489.81	194.34	0.40

miRNAs differentially expressed as compared to cirrhotic nodules also in HCV-unrelated pHCCs are indicated in bold.

Table 2. MiRNAs differentially expressed at all stages of HCC development compared to cirrhotic peritumoral tissue.

UniqueID	CIRR	LGDN	HGDN	eHCC	pHCC	FC LGDNC/CIRR	FC HGDNC/CIRR	FC eHCC/CIRR	FC pHCC/CIRR
Up-regulated miRNAs									
hsa-miR-664a-3p	9.03	215.77	212.24	266.27	177.48	23.89	23.50	29.49	19.65
hsa-miR-194-5p	217.6	9367.91	8375.23	3326.56	4177.61	43.05	38.49	15.29	19.20
hsa-miR-574-3p	14.34	356.54	369.8	374.84	258.14	24.86	25.79	26.14	18.00
hsa-miR-148a-3p	3380.01	41240.53	57007.13	29829.94	45111.2	12.20	16.87	8.83	13.35
hsa-miR-122-3p	95.74	3039.44	3018.01	768.65	1171.82	31.75	31.52	8.03	12.24
hsa-miR-126-5p	541.31	8003.48	8607.28	2839.93	6328.81	14.79	15.90	5.25	11.69
hsa-miR-16-5p	490.81	8703.37	8333.56	3565.24	5376.02	17.73	16.98	7.26	10.95
hsa-miR-26b-5p	755.48	14185.64	15893.82	6803.5	7899.58	18.78	21.04	9.01	10.46
hsa-miR-197-3p	18.48	276.11	250.41	190.24	189.87	14.94	13.55	10.29	10.27
hsa-miR-15a-5p	67	1196.72	1154.64	676.34	681.34	17.86	17.23	10.09	10.17
hsa-miR-32-5p	19.96	370.15	302.8	298.8	199.19	18.54	15.17	14.97	9.98
hsa-miR-21-5p	6004.4	61497.53	63932.67	44243.85	56869.68	10.24	10.65	7.37	9.47
hsa-miR-148b-3p	87.54	1077.73	1314.24	714.49	767.53	12.31	15.01	8.16	8.77
hsa-miR-26a-5p	3044.55	50356.74	46225.78	23954.32	25508.39	16.54	15.18	7.87	8.38
hsa-miR-30c-5p	206.2	3728.69	3949.79	1389.07	1685.54	18.08	19.16	6.74	8.17
hsa-miR-660-5p	52.67	495.08	446.94	258.77	427.34	9.40	8.49	4.91	8.11
hsa-miR-23b-3p	175.37	2068.46	2556.28	1312.82	1391.91	11.79	14.58	7.49	7.94
hsa-miR-24-3p	125.33	1444.8	1205.81	746.62	932.61	11.53	9.62	5.96	7.44
hsa-let-7f-5p	1312.47	17183.57	19460.83	8672.97	9728.21	13.09	14.83	6.61	7.41
hsa-miR-103a-3p	621.93	6532.97	4305.61	2482.1	3967.26	10.50	6.92	3.99	6.38
hsa-miR-224-5p	36.07	192.86	341.64	629.3	223.95	5.35	9.47	17.45	6.21
hsa-miR-151b	257.53	2202.92	2041.88	1110.19	1561.55	8.55	7.93	4.31	6.06
hsa-miR-151a-5p	259	2205.71	2046.57	1111.97	1564.01	8.52	7.90	4.29	6.04
hsa-let-7g-5p	698.84	6233.52	6668.44	3372.04	3676.97	8.92	9.54	4.83	5.26
hsa-let-7a-5p	1475.85	15346.63	15968.06	7800.91	7509.3	10.40	10.82	5.29	5.09
hsa-miR-101-3p	1660.08	15914.7	13266.34	7417.83	8219.51	9.59	7.99	4.47	4.95
hsa-miR-424-5p	89	954.28	890.09	794.8	437.34	10.72	10.00	8.93	4.91
hsa-miR-27b-3p	7334.8	54141.87	62761.76	28801.51	33296.42	7.38	8.56	3.93	4.54

Down-regulated miRNAs

hsa-miR-483-5p	1459.15	25.67	33.74	92.17	24.66	0.02	0.02	0.06
hsa-miR-1275	10031.5	55.99	38.69	137.67	173.34	0.01	0.00	0.01
hsa-miR-874-3p	1561.12	32.41	6.64	50.62	61.02	0.02	0.00	0.03
hsa-miR-320b	4334.96	108.69	59.01	208.91	179.72	0.03	0.01	0.05
hsa-miR-320a	37905.9	911.04	779.28	2053.3	1795.05	0.02	0.02	0.05
hsa-miR-3653-3p	1378.82	31.7	23.99	89.98	74.47	0.02	0.02	0.07
hsa-miR-138-5p	170.22	23.9	3.79	10.16	9.31	0.14	0.02	0.06
hsa-miR-4443	553.42	8.93	7.3	26.61	31.45	0.02	0.01	0.05
hsa-miR-320e	242.83	9.06	5.96	22.79	15.64	0.04	0.02	0.09
hsa-miR-375	6578.63	1338.6	476.8	1982.19	434.33	0.20	0.07	0.30
hsa-miR-320c	475.1	28.48	14.47	60.14	40.29	0.06	0.03	0.13
hsa-miR-320d	390.16	21.41	11.21	46.49	33.18	0.05	0.03	0.12
hsa-miR-1291	402.24	13.24	4.83	35.77	37.57	0.03	0.01	0.09
hsa-miR-769-5p	7721.65	289.11	232.8	739.02	817.5	0.04	0.03	0.10
hsa-miR-30c-2-3p	464.1	50.18	46.75	63.08	57.22	0.11	0.10	0.14
hsa-miR-127-3p	18801.6	917.14	815.22	1972.22	2732.21	0.05	0.04	0.10
hsa-miR-486-5p	18321.2	1706.43	2188.21	1711.34	2689.5	0.09	0.12	0.09
hsa-miR-1246	7273.51	486.89	326.15	1220.49	1070.49	0.07	0.04	0.17
hsa-miR-145-3p	1068.89	126.61	130.14	204.48	157.99	0.12	0.12	0.19
hsa-miR-1249-3p	100.64	11.02	9.88	15	16.24	0.11	0.10	0.15
hsa-miR-30c-1-3p	160.92	14.32	19.05	27.93	27.22	0.09	0.12	0.17
hsa-miR-146a-5p	8051.03	1316.45	711.7	1967.68	1382.08	0.16	0.09	0.24
hsa-miR-409-3p	1372.22	85.34	94.01	212.33	240.42	0.06	0.07	0.15
hsa-miR-29a-5p	187.78	21.25	27.59	40.88	33.68	0.11	0.15	0.22

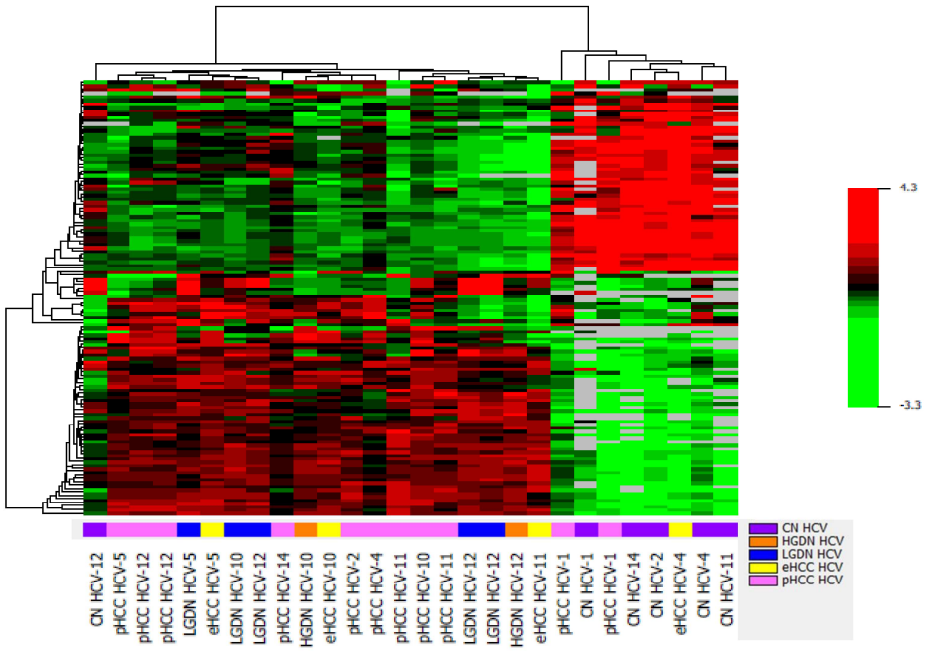


Fig. 1

Fig .2

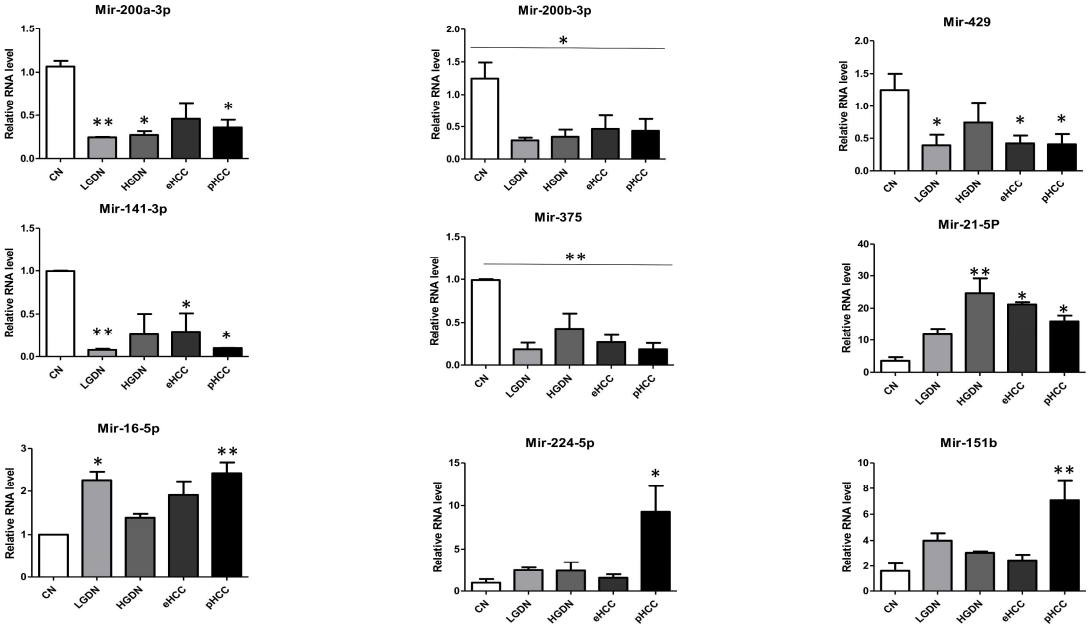
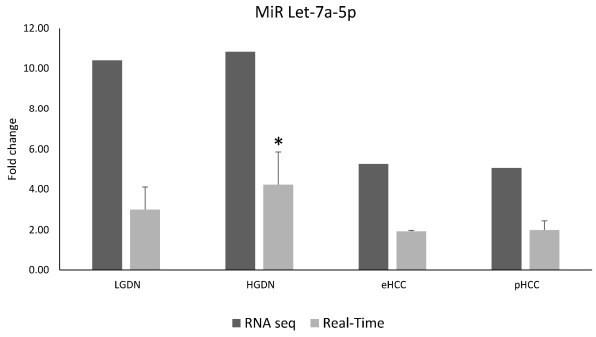
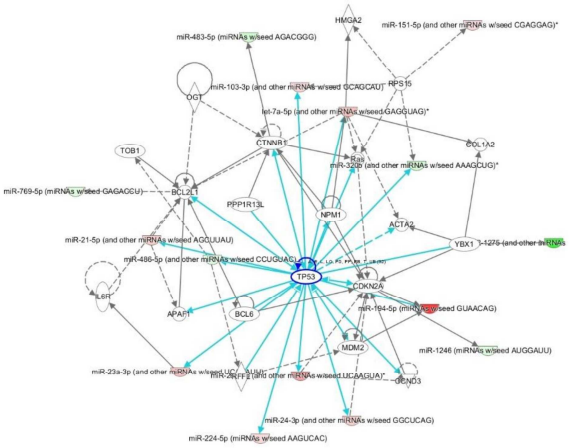


Fig. 3



A HGDN-LGDN



B pHCC

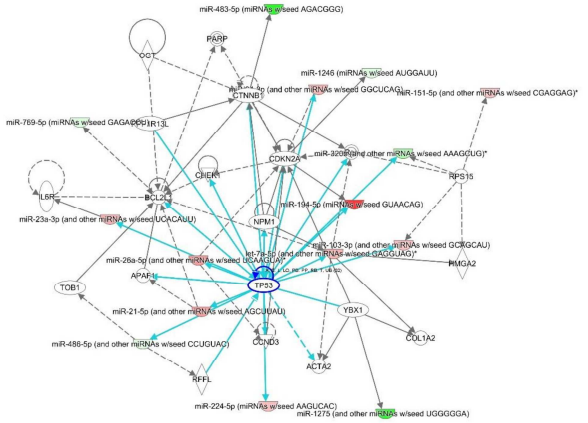


Fig. 4