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## **MicroRNA profiling in hepatocellular tumors is associated to clinical features and oncogene/tumor suppressor gene mutations**

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**Short title:** hepatocellular tumors miRNA profiling

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**Keywords:** Hepatocellular carcinoma, Hepatocellular adenoma, HBV, HCV, Alcohol consumption, Focal nodular hyperplasia, miRNA, Benign and malignant tumors, biomarkers, HNF1 $\alpha$ , microarray,  $\beta$ -catenin, transcriptome, liver cell adenoma.

Abbreviations: miRNA (microRNA), mRNA (messenger RNA) HCC (hepatocellular carcinoma), FNH (Focal Nodular Hyperplasia), HCA (hepatocellular adenoma), RT-PCR (Reverse Transcriptase Polymerase Chain Reaction), HBV (Hepatitis B virus), HCV (hepatitis C virus), siRNA (small interfering RNA), HNF1 $\alpha$  (hepatocyte nuclear factor 1 $\alpha$ ), PANK1 (pantothenate kinase 1).

**Abstract**

Molecular classifications defining new tumor subtypes has been recently refined using genetic and transcriptomic analyses of benign and malignant hepatocellular tumors. Here, we performed microRNA (miRNA) profiling in two series of fully annotated liver tumors to uncover associations between oncogene/tumor suppressors' mutations, clinical and pathological features. Expression levels of 250 miRNAs in 46 benign and malignant hepatocellular tumors were compared to 4 normal liver samples using quantitative RT-PCR. miRNAs associated to genetic and clinical characteristics were validated in a second series of 43 liver tumor and 16 non-tumor samples. miRNAs profiling unsupervised analysis classified samples in unique clusters characterized by histological features (tumor/non-tumor  $P < 0.001$ ; benign/malignant tumors  $P < 0.01$ , inflammatory adenoma and focal nodular hyperplasia  $P < 0.01$ ), clinical characteristics (HBV infection  $P < 0.001$ ; Alcohol consumption  $P < 0.05$ ) and oncogene/tumor suppressor gene mutations ( $\beta$ -catenin  $P < 0.01$ ; HNF1 $\alpha$   $P < 0.01$ ). Our study identified and validated miR-224 over-expression in all tumors, miR-200c, miR-200, miR-21, miR-224, miR-10b and miR-222 specific deregulation in benign or malignant tumors. Moreover miR-96 was over-expressed in HBV tumors, miR-126\* down regulated in alcohol related HCC. Down-regulations of miR-107 and miR-375 were specifically associated with HNF1 $\alpha$  and  $\beta$ -catenin gene mutations, respectively. miR-375 expression was highly correlated to that of  $\beta$ -catenin targeted genes as miR-107 expression was correlated to that of HNF1 $\alpha$  in a siRNA cell line model. Thus, it strongly suggests that  $\beta$ -catenin and HNF1 $\alpha$  could regulate miR-375 and miR-107 expression levels, respectively. *Conclusion:* Hepatocellular tumors may have distinct miRNAs expression fingerprint according to malignancy, risk factors and oncogene/tumor suppressor gene alterations. Dissecting these relationships provides new hypothesis to understand the functional impact of miRNAs deregulation in liver tumorigenesis and their promising use as diagnostic markers.

## Introduction

Hepatocellular adenomas (HCA) and hepatocellular carcinomas (HCC) are liver tumors heterogeneous in nature with various risk factors, genetics alterations, and clinical features (1-3). HCA are rare benign liver tumors usually developed in women after an oral contraceptive use (4). They are sometime difficult to discriminate from well-differentiated HCC or from focal nodular hyperplasia (FNH), a benign regenerative lesion. Recently, we defined a new molecular classification of HCA according to genetic (HNF1 $\alpha$  or  $\beta$ -catenin mutations) and phenotypic (inflammation) features (5, 6). We showed that  $\beta$ -catenin activated HCA were more at risk of malignant transformation than the other HCA subtypes.

Hepatocellular carcinoma (HCC) is the most common malignant tumor in the liver and the third cause of death from cancer. Viral (HBV and HCV infection) and non-viral (Alcohol and aflatoxin B1 ingestion) agents are known to be associated to HCC occurrence. In each tumor, several oncogenic pathways are deregulated (2) and different subtypes of tumors are defined according to genetic and transcriptomic based classification (7, 8). Briefly, two main tumor groups related to chromosome stability/instability were identified and six subgroups (called G1 to G6) were found closely related to genetic (*TP53* and  $\beta$ -catenin mutations), activation of oncogenic pathways (AKT and IGF activation), clinical features (local invasion), and risk factors (HBV exposure).

Apart of genetic and epigenetic abnormalities modifying oncogene and tumor suppressor genes, deregulation of microRNAs (miRNAs) may also contribute to carcinogenesis. miRNAs are small endogenous non-coding RNAs (~22 nucleotides) responsible for a post-transcriptional expression regulation of targeted genes. They promote mRNA degradation and repress mRNA translation by sequence-specific interaction with the 3'-untranslated region of targeted mRNAs (9). Physiologically, miRNAs have been shown to be involved in several processes such as development, apoptosis, proliferation and differentiation (10, 11). Moreover their levels of expression are deregulated in a large number of tumors, some of them were found directly implicated in carcinogenetic mechanisms and several altered expression of miRNAs has previously been described in rat and human HCC (12-14).

We hypothesized that miRNAs deregulation could be related to specific sub-groups of hepatocellular tumors defined by either oncogene/tumors suppressor mutations, histological or clinical features. We first analyzed the expression of 250 miRNAs in a series of 46 malignant and benign hepatocellular tumors extensively characterized compared to 4 normal liver tissues. According to tumor classification, the most significant deregulated miRNAs were

selected and their differential expression was validated in a second series of 43 tumors and 16 non-tumor liver tissues including cirrhosis and chronic hepatitis of various etiologies.

## Patients and Methods

### Liver Samples and Clinical Data

A whole series of 109 liver samples (55 HCC, 29 HCA, 5 Focal Nodular Hyperplasia, 30 non tumor liver samples) were collected from 93 patients surgically treated in Bordeaux from 1992 to 2004. Liver tissues were immediately frozen in liquid nitrogen and stored at -80°C until used for molecular studies. All the patients were recruited in accordance with French law and institutional ethical guidelines. Liver samples were fully clinically, histologically and genetically characterized and divided in two sets (**Table 1**). Hepatocellular adenomas (HCA) were classified according to the patho-molecular classification taking into account the HNF1 $\alpha$  or  $\beta$ -catenin mutations and the presence of inflammatory infiltrates (5, 6). HCC were classified according to the clinical, pathological and genetic features as classification previously described (7).

### Samples total RNAs extraction

Total RNAs extraction was performed using TRIZOL<sup>®</sup> Reagent (Invitrogen). A 2 mm<sup>3</sup> frozen tissue sample was homogenized in 800 $\mu$ l of TRIZOL<sup>®</sup> Reagent using a Tissue Lyser Mixer Mill MM 300 (Quiagen). The extraction was performed according to Invitrogen protocol. RNAs were quantified using NanoDrop<sup>®</sup> ND-1000 and quality was tested by migration of 200 ng RNA on 0.8% agarose gel.

### Quantitative RT-PCR

miRNAs quantitative RT-PCR was performed using TaqMan<sup>®</sup> MicroRNA Human Assays and the ABI PRISM<sup>®</sup> 7900HT System (Applied Biosystems). The first set of sample was analysed using a set of 250 miRNAs (Applied Biosystems, Supplementary Table 1). The expression of 22 miRNAs was further analysed in the validation set of tumors. For each sample and each miRNA tested, an independent retrotranscription using a specific reverse transcription primer was performed on 5 ng of totals RNAs according to manufacturer's protocol. Ribosomal 18s (R18S) was used for the normalization of expression data. The relative amount of miRNAs and all mRNAs studied in samples, was determined using the  $2^{-\Delta\Delta CT}$  method where  $\Delta\Delta CT = (CT_{\text{target}} - CT_{\text{R18S}})_{\text{sample}} - (CT_{\text{target}} - CT_{\text{R18S}})_{\text{calibrator}}$ . Briefly, for the first set of samples, expression of a miRNA was normalized with R18S and with the mean expression of the corresponding miRNAs in the 4 normal liver samples. For the validation set of samples, expression was normalized with R18s and with one normal liver sample. As the same normal liver RNA was previously included in the first set of experiment, we verified the reproducibility of miRNA expression values obtained in the two experiments. Results of the validated miRNA are provided in Supplementary Table 2.

HNF1 $\alpha$ /TCF1, GLUL, LGR5, TBX3 and PANK1 mRNA expression were measured as previously described (15) using pre-designed primers and probe sets from Applied Biosystems (probe set numbers: Hs00167041\_m1, Hs00374213\_m1, Hs00374213\_m1, Hs00255591\_m1, Hs00379740\_m1).

### **Clustering analysis**

In the first set of 50 samples, quantitative RT-PCR expression results for the 250 tested miRNAs were filtered, 130 miRNAs were selected on the basis of a Ct value below 31 in at least 10% of the tested samples (Supplementary Table 1). These 130 selected miRNA were used for an unsupervised clustering analysis. Dendrograms classifying samples and genes, with average linkage methods using Pearson correlation as a distance metric, was obtained based on the expression profile of quantitative RT-PCR using the dChip 2006 software (<http://biosun1.harvard.edu/complab/dchip/>). Clusters were considered significantly associated with a feature when the *P* value was less than 0.05 with Pearson correlation. miRNAs significantly differentially expressed according to clinical or genetic features identified in the unsupervised clustering analysis were selected for supervised analyses (Supplementary Table 3).

### **Statistical Analysis**

Statistical analysis was performed using dChip and GraphPad Prism softwares. Quantitative RT-PCR data were first analysed for differences of expression between samples subgroups using dChip t-test and the nonparametric Mann-Whitney test. miRNAs with a fold change superior to 3 and statistically significant in the two test were selected for validation (Supplementary Table 4). All miRNAs expression data were presented as mean  $\pm$  SEM in subgroups of tumors. Non-parametric Spearman test was used to compare quantitative values of expression. All reported *P* values were two-tailed and differences were considered significant when the *P* value was under 0.05.

### **Cell Culture and Transfections**

HepG2 cells were cultured in Dulbecco's modified Eagle Medium High Glucose (Invitrogen) supplemented with 10% fetal bovine serum, and penicillin/streptomycin. Cells were transfected using Lipofectamine RNAiMax (Invitrogen), in six wells plates, according to the manufacturer's protocol. HNF1 $\alpha$  extinction was performed with a siRNA targeting exon 8 and 9 of HNF1 $\alpha$  (siRNA 3544, sens :GGUCUUCACCUCAGACACUtt ; antisens : AGUGUCUGAGGUGAAGACCtg, Ambion). Several siRNA concentrations were tested (0, 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 5, 10, 50 nM) in order to modulate the HNF1 $\alpha$



expression. Maximal inhibition of HNF1 $\alpha$  protein was observed 72h post transfection (see Supplementary Fig. 1). BLOCK-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen) was used as transfected dsRNA control. Western blot was performed using HNF1 $\alpha$  antibody sc-6548 (Santa Cruz Biotechnology). At this time, cells were lysed in TRIZOL<sup>®</sup> Reagent (Invitrogen) for RNA extraction.

## Results

### **Global profiling of miRNAs is related to well defined-subgroups of hepatocellular tumors**

We analyzed the expression level of 250 miRNAs in the first set of samples that included 28 HCC, 13 HCA and 5 FNH compared to 4 normal liver tissues. An unsupervised analysis was performed after selection of the 130 miRNAs expressed in at least 10% of the samples (Fig. 1A). It showed a significant partition between non-tumor and tumor samples ( $P < 0.01$ ), benign tumors were divided in three clusters significantly parted from HCC ( $P < 0.01$ ). Adenomas were grouped in two distinct clusters corresponding to HNF1 $\alpha$  mutated ( $P < 0.01$ ) and Inflammatory HCA ( $P < 0.01$ ) respectively. Focal Nodular Hyperplasias were located on a third cluster ( $P < 0.01$ ) distinct from HCA. In contrast,  $\beta$ -catenin mutated HCA were spread between two HCA clusters. HCC samples were partitioned in three different clusters significantly characterized either by a specific clinical risk factor (HBV,  $P < 0.001$ ; Alcohol intake,  $P < 0.05$ ) or by a gene mutation ( $\beta$ -catenin,  $P < 0.01$ ). Two supervised analyses were performed to classify separately benign and malignant tumors using the list of genes differentially expressed in each clinical or genetic features significantly associated to clusters in the unsupervised analysis (Fig. 1B and C, Supplementary Table 3).

### **Specific miRNA deregulations related to benign or malignant features.**

As shown above, miRNA profiling could classify hepatocellular tumors according to pathological, clinical and genetic features. Thus, we aimed to identify and validate, in a second series of tumors, miRNA with a deregulated expression the most closely associated to such specific features. First, we identified miRNAs specifically deregulated in tumors when compared with non-tumor liver samples, and several were validated (Fig. 2). Three miRNAs were found significantly up-regulated (miR-224) or down-regulated (miR-422b and miR-122a) in both benign and malignant tumors (Fig. 2A). Second, we validated miRNAs with a level of expression significantly different between malignant and benign tumors. Corresponding to these criteria, miR-200c and miR-203 were under-expressed in benign tumors (Fig. 2B) while miR-21, miR-222 and miR-10b were significantly over-expressed in HCC (Fig. 2C). Finally, miR-224, that was differentially expressed between tumor and non-tumor liver tissues, was also significantly over-expressed in HCC when compared to benign tumors (Fig. 2A).

### **miRNAs deregulation related to tumor suppressor/oncogene mutations in HCA**

We searched for miRNAs deregulation that could characterize specific subgroups of benign tumors. While unsupervised clustering enabled to correctly classify inflammatory HCA and FNH in two specific clusters, no miRNA displayed a sufficient fold change of expression to

grant further validation. In contrast, a robust under expression of miR-375 was validated in  $\beta$ -catenin–mutated HCA when compared to the other HCA (fold change: -14.2,  $P < 0.001$  in overall samples, Fig. 3A). Among 15 miRNAs significantly deregulated in HNF1 $\alpha$ -mutated HCA, we validated the under expression of miR-107 in HNF1 $\alpha$  mutated HCAs when compared to the other HCA (fold change: -3.6;  $P < 0.001$ , in overall samples, Fig. 3B). This observation suggested that the HNF1 $\alpha$  transcription factor could regulate, directly or not, the expression of miR-107. To test this hypothesis, we performed an HNF1 $\alpha$  silencing using several doses of specific siRNA targeting HNF1 $\alpha$  in HepG2 hepatocellular cell line and we assayed the consequences on miR-107 expression level. The silencing of HNF1 $\alpha$  significantly correlated with the extinction of miR-107 in a dose-effect manner (Spearman  $r$ : -0.92;  $P < 0.0001$ , Fig. 3C). This result suggested that HNF1 $\alpha$  may control the expression of miR-107. MiR-107 is located in the intron 5 of *PANK1* gene coding for the pantothenate kinase 1. We tested if the miR-107 and *PANK1* expression could be co-regulated. We showed that *PANK1* mRNA expression was not affected by HNF1 $\alpha$  silencing, suggesting that regulation of miR-107 by HNF1 $\alpha$  was independent of *PANK1* status (Supplementary Fig. 2). According to the hypothesis of a direct regulation of miR-107 by HNF1 $\alpha$ , the *in silico* search (using Genomatix softwares) revealed two potential HNF1 $\alpha$  binding sites located -1852 and -1543 base pairs upstream of the pre-miRNA initiation site. However, the direct role of HNF1 $\alpha$  remained to be fully demonstrated by a functional analysis of the putative promoter region.

### **miRNAs deregulation related to genetic features and risk factors in HCC**

As shown in Fig. 1, miRNAs deregulations were associated with alcohol consumption and HBV infection in HCC. We validated miR-126\* under-expression in HCC related to alcohol abuse when compared to the other HCC (fold change: 2.7;  $P < 0.001$ , in overall samples, Fig. 4A). Similarly, we also validated a significant over-expression of miR-96 in HBV related HCC when compared to non-infected tumors (fold change: 5.7;  $P < 0.01$ , in overall samples, Fig. 4B). In contrast, the level of expression of miR-126\* and miR-96 did not vary significantly among the various subtypes of non-tumor liver tissues (Supplementary Fig. 3). In particular, no significant variation was observed in cirrhosis or in HBV related non-tumor samples. As suggested by the unsupervised clustering, no miRNA were found significantly deregulated in HCCs HCV infected versus non-infected HCCs. Similarly, no significant difference was found according to the level of cell differentiation.

microRNAs deregulations were also related to the  $\beta$ -catenin oncogene mutation in the unsupervised clustering analysis (Fig 1). As in  $\beta$ -catenin mutated HCAs, we validated the extinction of miR-375 in HCC characterized by a  $\beta$ -catenin activating mutation (fold change: -44;  $P < 0.001$ , in overall samples, Fig. 4C). We also found a significant anti-correlation

between the level of expression of miR-375 and three genes regulated by  $\beta$ -catenin, *TBX3*, *LGR5* and *GLUL* in HCA as well in HCC (Fig. 4D). Finally, no significant differences in miRNA expression was observed in non-tumor liver tissues according to HCV or HBV infection, alcohol intake and cirrhosis.

## Discussion

Aberrant patterns of miRNAs expression have already been described in several hematological and solid tumors, including HCC (see for review (16)). In the present work, we have identified, for the first time, miRNAs expression patterns that can unambiguously differentiate benign, malignant and several subtypes of hepatocellular tumors according to specific risk factors, oncogene and tumor suppressor gene mutations. These new findings and the most significant deregulated miRNAs are summarized in Fig. 5.

In the present work, several miRNAs previously associated with malignancy were identified as significantly deregulated in hepatocellular subgroup of tumors. Most notably, we showed that miR-224 previously identified as over-expressed in HCC (14) was also up-regulated in benign hepatocellular tumors, including adenomas and focal nodular hyperplasia that are regenerative polyclonal nodules (17), but at a lesser degree. Similarly, miR-122a has been previously found down-regulated in human and rodent HCC (12); this microRNA, highly expressed in the liver, is essential for HCV replication (18) and it modulates cyclin G1 expression in HCC cell-line (19). Here, we showed that miR-122a is also down-regulated in both human benign and malignant hepatocellular tumors. Thus, concomitant analysis of benign and malignant tumors enabled us to associate deregulation of expression of miR-224, 422b and 122a with common early events in hepatocellular tumorigenesis.

Only few miRNAs deregulations have been previously described in benign tumors (20, 21). Interestingly, in pituitary adenoma, miRNAs pattern of expression were significantly related to tumor subtype according to histotypes and secreting characteristics (20). In thyroid nodules, most of the miRNAs deregulated in carcinomas have demonstrated also an altered expression in adenomas (21). Here, we identified miRNA (miR-200c and miR-203) with an expression specifically down-deregulated in benign hepatocellular tumors when compared to normal liver tissues or HCC. A lower expression of miR-203 was also previously described in non-secreting pituitary adenomas when compared with growth hormone pituitary secreting adenomas (20). In hepatocellular tumors, we showed that miRNAs profile of expression in benign tumor differed according to tumor subtype defined by oncogene and tumor suppressor gene mutation or by histological phenotype. These results enlightened the importance of refining classification based on molecular and meticulous characterization of the tumor phenotypes, including for benign tumors.

Here we validated a significant over-expression of miR-10b, miR-21 and miR-222 in HCC when compared with benign tumors and non-tumor liver tissues. Interestingly, over-expression of these three miRNAs was previously identified as important key factors to promote cell invasion or proliferation in various epithelial tumor types. Recently, Ma and collaborators identified miR-10b as a microRNA highly expressed in metastatic breast tumors

that promotes cell migration and invasion (22). They showed that miR-10b expression is induced by twist allowing miR-10b to inhibit translation of the messenger RNA encoding homeobox D10, resulting in increased expression of a well-characterized pro-metastatic gene, RHOC. miR-222 over expression promotes cell proliferation by targeting p27Kip1 in prostate and thyroid carcinoma (23, 24). Finally, miR-21 over-expression down-regulates the Pdc4 tumor suppressor and stimulates invasion, intravasation and metastasis in colorectal cancer (25). Higher over-expression of miR-21 was also previously associated with poorly differentiated HCC and it participates to down-regulate the level of expression of PTEN (13).

Our results identified the deregulation of two miRNAs, miR-96 and miR-126\*, as significantly associated with different risk factors of HCC, HBV infection and alcohol consumption, respectively. To our knowledge, miR-96 and miR-126\* have not been directly implicated in pathways related to carcinogenesis. Because no significant variation in the expression of these two microRNAs was observed in non-tumor tissues according to HBV infection or alcohol intake, it suggests that microRNAs deregulations are directly linked to carcinogenetic processes specifically induced by these two risk factors. In fact, using transcriptomic and genetic global analyses we have previously shown that carcinogenesis mechanisms in HBV related HCC were particular, closely related to chromosome instability, AKT activation and fetal features in gene expression (7, 8). In contrast, microRNA profiling is the first global genomic approach that enabled to discriminate HCC related to alcohol abuse from the other tumors. Consequences of the miR-126\* deregulation remained to be explored in alcohol related tumors and HCC associated to other risk factors.

For the first time, we identified miRNAs deregulation in hepatocellular human tumors closely related to specific oncogene or tumor suppressor gene mutations. However, in cell line models, some miRNAs have been shown to be under control of oncogene (27) or tumor suppressor (28). The low expression of miR-375 in both HCA and HCC mutated for  $\beta$ -catenin together with the highly significant anti-correlation between miR-375 level of expression and genes targeted by  $\beta$ -catenin argue for a direct link between  $\beta$ -catenin activation and repression of miR-375. This is an important result since  $\beta$ -catenin is a major oncogene activated in 30 to 40% of the HCC. It increases the complexity of regulation of expression by  $\beta$ -catenin adding yet another layer of control with microRNA to the RNA and translation control level. In HCA, we showed a specific under-expression of miR-107 in HNF1 $\alpha$  inactivated samples. It has been suggested by *in silico* analysis that miR-107 could regulate the expression level of several predicted target proteins involved in lipid metabolism. Under-expression of miR-107 is predicted to promote acetyl-coA and lipids levels (26). Interestingly, we previously showed that HNF1 $\alpha$  inactivated HCA exhibited a steatotic phenotype resulting from an activation of lipogenesis (29). Thus, we can hypothesize that miR-107 under-

expression specifically observed in HNF1 $\alpha$  inactivated HCA may contribute to the steatotic phenotype and lipid accumulation. Our data suggest that this miRNA could be regulated by HNF1 $\alpha$  like the recently identified miR-194 during intestinal epithelial Caco-2 cell line differentiation (30).

In conclusion, this work revealed an additional layer of gene alterations to better characterize hepatocellular tumors. It allowed the identification of miRNAs deregulation closely correlated to clinical and histological subtypes of tumors as well as to oncogene and tumor suppressor gene mutations. The gene and protein networks directly targeted and affected by these miRNAs that participate to tumorigenesis remain to be extensively explored.

## References

1. El-Serag HB. Hepatocellular carcinoma: recent trends in the United States. *Gastroenterology* 2004;127:S27-34.
2. Laurent-Puig P, Zucman-Rossi J. Genetics of hepatocellular tumors. *Oncogene* 2006;25:3778-3786.
3. Villanueva A, Newell P, Chiang DY, Friedman SL, Llovet JM. Genomics and signaling pathways in hepatocellular carcinoma. *Semin Liver Dis* 2007;27:55-76.
4. Baum JK, Bookstein JJ, Holtz F, Klein EW. Possible association between benign hepatomas and oral contraceptives. *Lancet* 1973;2:926-929.
5. Bioulac-Sage P, Rebouissou S, Thomas C, Blanc JF, Saric J, Sa Cunha A, Rullier A, et al. Hepatocellular adenoma subtype classification using molecular markers and immunohistochemistry. *Hepatology* 2007;46:740-748.
6. Zucman-Rossi J, Jeannot E, Nhieu JT, Scoazec JY, Guettier C, Rebouissou S, Bacq Y, et al. Genotype-phenotype correlation in hepatocellular adenoma: new classification and relationship with HCC. *Hepatology* 2006;43:515-524.
7. Boyault S, Rickman DS, de Reynies A, Balabaud C, Rebouissou S, Jeannot E, Herault A, et al. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. *Hepatology* 2007;45:42-52.
8. Laurent-Puig P, Legoix P, Bluteau O, Belghiti J, Franco D, Binot F, Monges G, et al. Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. *Gastroenterology* 2001;120:1763-1773.
9. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281-297.
10. Ambros V. The functions of animal microRNAs. *Nature* 2004;431:350-355.
11. Miska EA. How microRNAs control cell division, differentiation and death. *Curr Opin Genet Dev* 2005;15:563-568.
12. Kutay H, Bai S, Datta J, Motiwala T, Pogribny I, Frankel W, Jacob ST, et al. Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. *J Cell Biochem* 2006;99:671-678.
13. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* 2007;133:647-658.
14. Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, Shimotohno K. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *Oncogene* 2006;25:2537-2545.



15. Rebouissou S, Vasiliu V, Thomas C, Bellanne-Chantelot C, Bui H, Chretien Y, Timsit J, et al. Germline hepatocyte nuclear factor 1alpha and 1beta mutations in renal cell carcinomas. *Hum Mol Genet* 2005;14:603-614.
16. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857-866.
17. Rebouissou S, Bioulac-Sage P, Zucman-Rossi J. Molecular pathogenesis of focal nodular hyperplasia and hepatocellular adenoma. *J Hepatol* 2007.
18. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 2005;309:1577-1581.
19. Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, Calin GA, et al. Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res* 2007;67:6092-6099.
20. Bottoni A, Zatelli MC, Ferracin M, Tagliati F, Piccin D, Vignali C, Calin GA, et al. Identification of differentially expressed microRNAs by microarray: a possible role for microRNA genes in pituitary adenomas. *J Cell Physiol* 2007;210:370-377.
21. Weber F, Teresi RE, Broelsch CE, Frilling A, Eng C. A limited set of human MicroRNA is deregulated in follicular thyroid carcinoma. *J Clin Endocrinol Metab* 2006;91:3584-3591.
22. Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* 2007;449:682-688.
23. Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafre SA, Farace MG. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. *J Biol Chem* 2007;282:23716-23724.
24. Visone R, Russo L, Pallante P, De Martino I, Ferraro A, Leone V, Borbone E, et al. MicroRNAs (miR)-221 and miR-222, both overexpressed in human thyroid papillary carcinomas, regulate p27Kip1 protein levels and cell cycle. *Endocr Relat Cancer* 2007;14:791-798.
25. Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdc4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene* 2007.
26. Wilfred BR, Wang WX, Nelson PT. Energizing miRNA research: a review of the role of miRNAs in lipid metabolism, with a prediction that miR-103/107 regulates human metabolic pathways. *Mol Genet Metab* 2007;91:209-217.
27. Schulte JH, Horn S, Otto T, Samans B, Heukamp LC, Eilers UC, Krause M, et al. MYCN regulates oncogenic MicroRNAs in neuroblastoma. *Int J Cancer* 2007;122:699-704.
28. He L, He X, Lowe SW, Hannon GJ. microRNAs join the p53 network--another piece in the tumour-suppression puzzle. *Nat Rev Cancer* 2007;7:819-822.

29. Rebouissou S, Imbeaud S, Balabaud C, Boulanger V, Bertrand-Michel J, Terce F, Auffray C, et al. HNF1alpha inactivation promotes lipogenesis in human hepatocellular adenoma independently of SREBP-1 and carbohydrate-response element-binding protein (ChREBP) activation. *J Biol Chem* 2007;282:14437-14446.
30. Hino K, Fukao T, Watanabe M. Regulatory interaction of HNF1-alpha to microRNA-194 gene during intestinal epithelial cell differentiation. *Nucleic Acids Symp Ser (Oxf)* 2007:415-416.

### Figures:

Fig. 1. (A) Unsupervised hierarchical clustering of miRNA profiling in 50 liver samples. The dendrogram in the upper panel was obtained on the basis of the quantitative RT-PCR data of the 130 miRNAs expressed in at least 10% of the liver samples that included 4 normal livers, 28 HCC, 13 HCA and 5 FNH. (B) Supervised clustering analysis of miRNA significantly expressed according to each sub-group (see Supplementary Table 3) in the 18 benign tumors samples. (C) Supervised clustering analysis of miRNA differentially expressed in  $\beta$ -catenin mutated HCC, alcohol related HCC and HBV infected HCC (see Supplementary Table 3) in the 28 HCC samples. The different significant tumors subgroups are indicated in color. The heat map, in the lower panel, shows the expression of miRNAs across samples. For each miRNA probeset, data are median-centered (black), with the lowest and highest intensity values in green and red, respectively.

Fig. 2. miRNAs the most significantly deregulated in hepatocellular tumors. (A) miR-224 was found over-expressed, miR-422b and miR-122a were found down regulated in tumors when compared to non tumor liver. (B) miR-200c and 203 were found under-expressed in benign tumors when compared to HCC. (C) miR-21, 222 and 10b were found over-expressed in HCC when compared to benign tumors. FS=First Set of tumors, VS=Validation Set of tumors. B=Benign tumors (FS n=18; VS n=12); HCC=Hepatocellular Carcinomas (FS n=28; VS n=22); NL=Normal Livers (n=4), NTL=Non Tumor Liver (n=16, including cirrhosis n=9; HBV infected n=6; HCV infected samples n=5; alcohol liver disease n=5). Data are presented as the mean fold compared to the mean value in non tumor samples  $\pm$  SEM. Statistical analysis was performed using Mann-Whitney test, \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001.

Fig. 3. miRNAs deregulation associated to tumor suppressor gene or oncogene mutation in HCA. (A) miR-375 was under-expressed in  $\beta$ -catenin mutated HCA ( $\beta$ -c Mut; FS n=5, VS

n=4), versus non mutated HCA ( $\beta$ -c Wt; FS n=13, VS n=8). (B) miR-107 was found down-expressed in HNF1 $\alpha$  mutated HCA (H $\alpha$  Mut; FS n=4; VS n=7), versus non mutated HCA (H $\alpha$  Wt; FS n=9, VS n=8). For Normal Liver (NL) and Non Tumor Liver (NTL) distribution, see Fig. 2. (C) Correlation between miR-107 and HNF1 $\alpha$  transcript level of expression in HepG2 cell line. The indicated miR-107 expression is the observed miR-107 expression divided by the level of miR-107 expression in basal HNF1 $\alpha$  condition. Statistical analysis by Spearman test.

Fig. 4. miRNAs deregulation associated to risk factors and oncogene mutation in HCC. (A) miR-126\* was under-expressed in HCC related to alcohol abuse (Al+; FS n=7; VS n=6) vs others HCC (Al-; FS n=10, VS n=8). (B) miR-96 was over-expressed in Hepatitis B Virus infected HCC (HBV+; FS n=9; VS n=9) versus non infected HCC (HBV-; FS n=10; VS n=8). (C) miR-375 is down expressed in  $\beta$ -catenin mutated HCC ( $\beta$ -c Mut; FS n=7, VS n=8) versus non mutated ( $\beta$ -c Wt; FS n=21, VS n=14) HCC. For Normal Liver (NL) and Non Tumor Liver (NTL) distribution, see Fig. 2. (D) Correlation between miR-375 and three genes regulated by  $\beta$ -catenin in hepatocellular tumors. FS=First Set of tumors, VS=Validation Set of tumors.

Fig. 5. miRNAs expression is associated to different subgroups of hepatocellular tumors. HCC: Hepatocellular Carcinomas; HCA: Hepatocellular Adenomas;  $\uparrow$ : over-expression;  $\downarrow$ : under-expression

### Supplementary Figures:

Supplementary Fig. 1. Inhibition of HNF1 $\alpha$  protein expression was shown 72 hours after transfection of HepG2 by 10 nM of a specific siRNA (see Patients and methods).

Supplementary Fig. 2. *PANK1* expression is not correlated with HNF1 $\alpha$  expression when HNF1 $\alpha$  expression was modulated by siRNA.

Supplementary Fig. 3. miR-126\* and miR-96 expression in non tumor liver (NTL). Non Cirrhotic NTL (NTL Non Cirr; n=7), Cirrhotic NTL (NTL Cirr; n=9), NTL non exposed to alcohol (NTL Alcohol-; n=11), NTL exposed to alcohol (NTL Alcohol+; n=5), NTL non infected by HBV (NTL HBV-; n=10), NTL infected by HBV (NTL HBV+; n=6).