

Downregulation of miR-122 in the Rodent and Human Hepatocellular Carcinomas

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Abstract MicroRNAs (miRs) are conserved small non-coding RNAs that negatively regulate gene expression. The miR profiles are markedly altered in cancers and some of them have a causal role in tumorigenesis. Here, we report changes in miR expression profile in hepatocellular carcinomas (HCCs) developed in male Fisher rats-fed folic acid, methionine, and choline-deficient (FMD) diet. Comparison of the miR profile by microarray analysis showed altered expression of some miRs in hepatomas compared to the livers from age-matched rats on the normal diet. While *let-7a*, *miR-21*, *miR-23*, *miR-130*, *miR-190*, and *miR-17-92* family of genes was upregulated, *miR-122*, an abundant liver-specific miR, was downregulated in the tumors. The decrease in hepatic miR-122 was a tumor-specific event because it did not occur in the rats switched to the folate and methyl-adequate diet after 36 weeks on deficient diet, which did not lead to hepatocarcinogenesis. miR-122 was also silent in a transplanted rat hepatoma. Extrapolation of this study to human primary HCCs revealed that miR-122 expression was significantly ($P=0.013$) reduced in 10 out of 20 tumors compared to the pair-matched control tissues. These findings suggest that the downregulation of miR-122 is associated with hepatocarcinogenesis and could be a potential biomarker for liver cancers. *J. Cell. Biochem.* 99: 671–678, 2006. © 2006 Wiley-Liss, Inc.

Key words: folate/methyl-deficient diet; hepatocellular carcinoma; microRNA; miR-122; miR-17-92; miR-21

Micro RNAs (miRs)¹ are small, non-coding RNA molecules identified in plants, animals, and viruses and are primarily involved in gene silencing (for review, see Bartel [2004]; Du and Zamore [2005]). Several hundred miRs have been cloned and sequenced (see [\[rna.sanger.ac.uk\]\(http://micro-rna.sanger.ac.uk\)\). In animals, these are evolutionarily conserved, 21–26 mer RNAs that block translation by imperfect base pairing to the 3'-untranslated regions \(3'-UTR\) of specific mRNA and by inducing mRNA degradation. Most miRs are expressed as primary transcripts transcribed by pol II, some miRs in clusters are coordinately expressed, and others are generated from introns. Primary miRs \(pri-miRs\) have 5' caps and 3' poly \(A\) tails, which is processed to mature miR by specific ribonuclease complexes \(for review, see Zeng et al. \[2005\]\). miRs play a key role in regulating diverse cellular processes that include development, differentiation, cell growth, apoptosis, viral infection, and metabolism \(for review, see Ambros \[2004\]\).](http://micro-</p></div><div data-bbox=)

Like mRNAs, the majority of miRs are expressed predominantly in a tissue-specific manner whereas some are enriched in certain tissue [Lagos-Quintana et al., 2002]. Recently, much attention has been focused on miRs and

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cancer since miR genes are located at chromosomal regions, characterized by fragile sites and regions of deletion or amplification [Calin et al., 2004]. Some of these miRs deregulated in cancer function as tumor suppressors or oncogenes (for review, see Hwang and Mendell [2006]).

Our laboratory has been studying the transcriptional and epigenetic regulation of gene expression in rodent and human primary hepatocellular carcinomas (HCCs) [Majumder et al., 2002; Ghoshal et al., 2004]. To study the altered regulation of gene expression at different stages of hepatocarcinogenesis, we have used a rat model. In this model, Fisher male rats fed folate and methyl-deficient (FMD) diets develop preneoplastic nodules after 36 weeks and HCCs after 54 weeks [Motiwala et al., 2003; Li, 2006]. Recently, we have used this rat model to identify the genes that are regulated tumor-specifically by epigenetic mechanism [Motiwala et al., 2003]. This model system was ideal to study alteration in miR expression during multistage hepatocarcinogenesis. In the present study, we used microarray analysis to identify miRs that are deregulated in the hepatomas induced by folate/methyl deficiency. Among these, miR-122, a liver-specific miR, was suppressed not only in rodent but also in human HCCs.

MATERIALS AND METHODS

Animals and Diet

The dietary regimen has been described in detail earlier [Pogribny et al., 1995; Motiwala et al., 2003]. Four-week-old male Fisher 344 rats were fed diet low in L-methionine (0.18%), devoid of choline and folic acid (FMD) or the methyl-adequate diet (deficient diet supplemented with 0.4% L-methionine, 0.3% choline, and 2 mg/kg folic acid). Four rats from each group were sacrificed after 9, 18, 36, or 54 weeks. No significant differences in body weight and food consumption were noted between the diet groups. RNA was isolated from the frozen livers as described [Ghoshal et al., 2004].

MicroRNA Microarray

Total RNA from three HCC samples and three age-matched livers from rats on normal diet were used for microarray analysis. RNA labeling and hybridization on miR microarray chips was done as described [Volinia et al., 2006]. Microarray was performed at Ohio State Uni-

versity Comprehensive Cancer Center Microarray core facility. Since miRs are conserved, we used microarrays that contain both human and mouse miR genes to profile rat miRs. Briefly, 5 μ g of RNA from each sample was biotin-labeled during reverse transcription using random hexamers. Hybridization was carried out on miR microarray chip (KCI version 1.0), which contains 368 probes, including 245 human and mouse miR genes (both precursors and mature), in duplicate. Each sample was hybridized to duplicate array. Hybridization signals were detected by biotin binding of a streptavidin–Alexa 647 conjugate using a Perkin–Elmer ScanArray XL5K. Scanned images were quantified by the Quantarray software (Perkin–Elmer, Wellesley, MA).

Statistical and Bioinformatic Analysis of Microarray Data

Raw data were normalized and analyzed using the Microsoft Excel software. The signal in each miR was normalized to the median of all miRs in each array to allow comparison among different arrays. Hierarchical clustering was done using spearman rank correlation with complete linkage analysis and TREEVIEW. Statistical analysis was performed using ANOVA and student *t*-test.

Northern Blot Analysis

Total RNA was separated on a denaturing (7 M urea) polyacrylamide (15% acrylamide) gel, electroblotted to nylon membrane, and fixed to the membrane by heating at 80°C for 2 h. It was prehybridized at 42°C in a buffer containing 5 \times SSPE, 2 \times Denhardt's solution, 7% SDS, and 100 μ g/ml salmon sperm DNA and hybridized overnight at 42°C in the same buffer containing ³²P-labeled deoxyoligonucleotide (1.5 \times 10⁶ cpm/ml) antisense to a specific miR [Jiang et al., 2005]. The sequences of the antisense oligos are complimentary to those of mature miRs (available from <http://microrna.sanger.ac.uk>). The blot was washed thrice for 30 min at 42°C with buffer 1 (3 \times SSPE, 5% SDS, 10 \times Denhardt's solution, and 50 μ M ATP) and once for 5 min in buffer 2 (1 \times SSC and 1% SDS). The membrane was subjected to autoradiography and phosphorimager analysis and the signal quantified using volume-analysis program. The statistical analysis was performed using student *t*-test.

RESULTS

MicroRNA Expression Profile Is Altered in Hepatocellular Carcinomas Developed in Rats-Fed FMD Diet

Microarray analysis was performed to examine the potential changes in miR levels during FMD diet-induced hepatocarcinogenesis in rats. The expression of each miR was normalized to the average median of all the genes in an array. We eliminated miRs whose levels were equal to or below the negative controls (bacterial plasmids and some ultraconserved sequences) and those that did not have significant difference among the three livers (N) and three tumors (T). The median-normalized signal for each miR (spotted in duplicate in each array) in a specific sample (liver or tumor) was comparable among duplicate arrays. Unsupervised clustering of miRs based on the expression data revealed non-random classification of the samples into two groups; three normal livers clustered together and were well separated from the tumors that form a distinct group from the controls (Fig. 1).

To identify the differentially expressed miRs in HCCs, the ratio of the signal for each miR to that of the average value of three normal livers (controls) was determined. The expression profile of most of the miRs was comparable among three controls. We selected those miRs that showed twofold or higher expression in at least two out of three tumors analyzed compared to the controls. The same analysis was performed to identify all miRs underexpressed (ratio = 50%) in at least two tumors. Classification of 245 miRs based on the expression profile identified 23 upregulated and only three downregulated miR genes (Table I). This observation is consistent with a recent study showing the upregulation of miR genes is more common than downregulation in solid tumors in microarray-based expression profiling [Volinia et al., 2006]. Among the 23 upregulated miRs, 9 (miR-101b-2, -130-130a, -172a-2, -219-1, -23a, -23b, -24, and -328-1) were elevated twofold or higher in all three tumors (Table I). Strikingly, only miR-122, a liver-specific miR, was reduced by 50% in all three tumors whereas miR-123 and miR-215 levels decreased in two out of three tumors. Further K-mean analysis revealed that two groups of miRs are either up or downregulated in tumors compared to all the controls (Table II). As the sample size was relatively

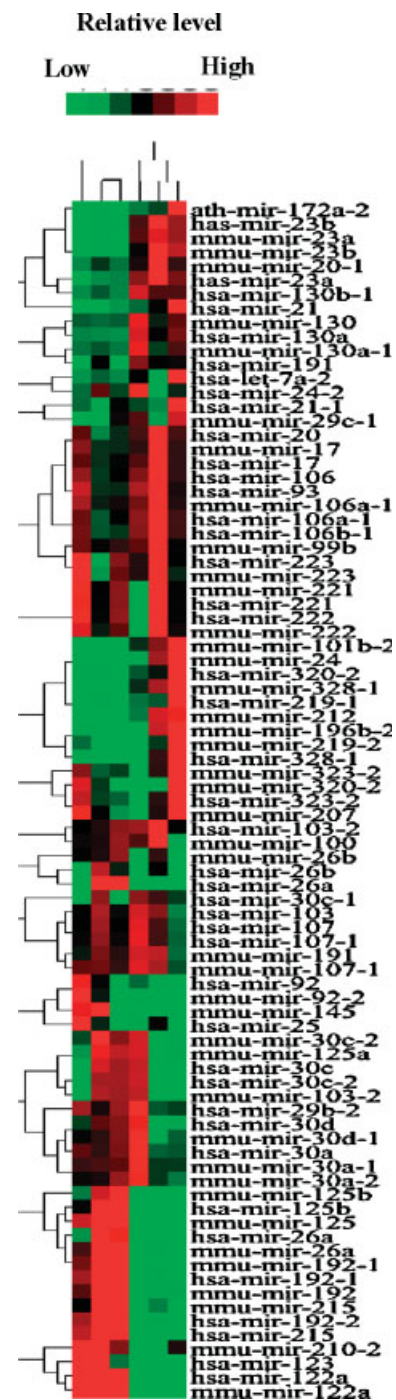


Fig. 1. Cluster analysis of three HCCs and three age-matched rat livers. Microarray analysis of miRs expressed in the rat livers and hepatocellular carcinomas (HCCs). miR expression data were normalized to the average median of all the genes in the array. Only those miRs were used for clustering whose normalized value are higher than 1.5 and their expression were significantly different (ratio = 1.5, OR = 0.5) in at least one HCC and age-matched rat liver. N and T stands for normal liver and tumor, respectively.

TABLE I. Differentially Regulated miRs in HCC

miRNA	T1/N	T2/N	T3/N
Upregulated			
miR-101b-2	3.7	2.7	3.1
miR-130	5.9	2.5	2
miR-130a	5.7	2.6	2.2
miR-172a-2	3.6	2.4	3.5
miR-219-1	2.9	2	2.4
miR-23a	4.1	2.8	2.2
miR-23b	4.6	3.8	2.6
miR-24	3.4	2.9	3.1
miR-328-1	3.8	2.9	2.9
let-7a-2	3.9	1.8	3.7
miR-103-2	3.1	2.2	1
miR-106	3.6	2.7	1.3
miR-106a-1	3.8	2.8	1.4
miR-106b-1	2.9	2.2	1.3
miR-130a-1	5.4	2.2	1.9
miR-17	4.4	3.7	1.5
miR-20	4.7	3.9	1.7
miR-20-1	3.1	2.9	1.5
miR-21	2.4	1.8	2.4
miR-21-1	2.3	1.4	2.5
miR-320-2	2.3	1.6	2.4
miR-93	3	2.1	1.2
miR-99b	4.8	3.8	1.2
Downregulated			
miR-122	0.4	0.3	0.2
miR-123	0.9	0.5	0.4
miR-215	0.5	0.9	0.4

The expression in each tumor was normalized to the average value of all three normal livers. We listed only those genes that are upregulated \geq twofold or downregulated \geq 50% in at least two tumors. T1, T2, and T3 are the ratio of expression in each tumor, which was normalized to that of the average of three livers (N).

small we considered P -value = 0.1 as significant. It is notable that more downregulated miRs (miR-192, -26a, and -125) were detected using this analysis (Table II). Importantly, we observed consistent results for the majority of the miRs with both human and mouse miR probes.

Northern Blot Analysis Confirmed Differential Regulation of Expression of Some miRs During FMD Diet-Induced Hepatocarcinogenesis

We next validated microarray data by Northern blot analysis of a few of the differentially

TABLE II. P-Values of miRs Differentially Regulated in HCCs as Determined by *t*-Test

	<i>P</i> -value
Upregulated	
miR-17	0.06
miR-20	0.04
miR-21	0.02
miR-93	0.04
miR-101b-2	0.02
miR-106	0.05
miR-106a-1	0.04
miR-106b-1	0.03
miR-130	0.07
miR-130a	0.04
miR-130a-1	0.07
miR-130b-1	0.02
miR-17-1	0.06
miR-17-2	0.06
miR-172a-2	0.07
miR-20-1	0.07
miR-212	0.04
miR-219-1	0.09
miR-24	0.04
miR-320-2	0.09
miR-328-1	0.01
miR-23a	0.02
miR-23b	0.02
let-7a-2	0.10
Downregulated	
miR-122	0.02
miR-123	0.04
miR-125b-1	0.06
miR-125b-2	0.05
miR-192-1	0.07
miR-192-2	0.08
miR-215	0.08
miR-26a-1	0.09
miR-26a-2	0.07
miR-26a	0.09

miRs with $P = 0.1$ were considered significant, as our sample size was relatively small (three in each group).

expressed miRs. Additionally, we also compared the expression of these miRs in the livers of rat-fed FMD diet for 36 weeks when preneoplastic changes take place [Motiwala et al., 2003]. miR-122 expression decreased in all four HCCs compared with the control livers but not in the PNNs (Fig. 2A,B). As expected, the liver-specific miR-122 was not detectable in the brain. The reduced expression of miR-122 probably occurred between 36 and 54 weeks when neoplastic transformation occurs. It is noteworthy that the diet-induced downregulation of hepatic miR-122 did not occur in the rats switched to

Fig. 2. A: Northern blot analysis confirmed downregulation of miR-122 and upregulation of miR-23, -21 -130, and let-7 in HCC compared with the controls. Total RNA was isolated from the livers of four rats-fed normal diet, four rats-fed FMD diet for 36 and 54 weeks, respectively. An aliquot (30 μ g) of the total RNA was separated by denaturing PAGE, transferred to a nylon membrane and subjected to Northern blot analysis with 32 P-labeled deoxyoligonucleotide antisense to specific miRs. The blot was reprobed with oligo antisense to 5S rRNA and the ratio of

miR signals to that of 5S rRNA were determined. **B:** Quantitative analysis of Northern blot data showed significant downregulation of miR-122 and upregulation of miR-21, -23, and -130 and let-7 in rat HCCs. 32 P-signal was measured using Imagequant software and quantified using volume analysis program. PNN stands for preneoplastic nodules. The results represent the average signal of each miR normalized to that of 5S rRNA \pm SD. P -value of ≤ 0.05 was considered significant. Asterisks indicate changes that are significant.

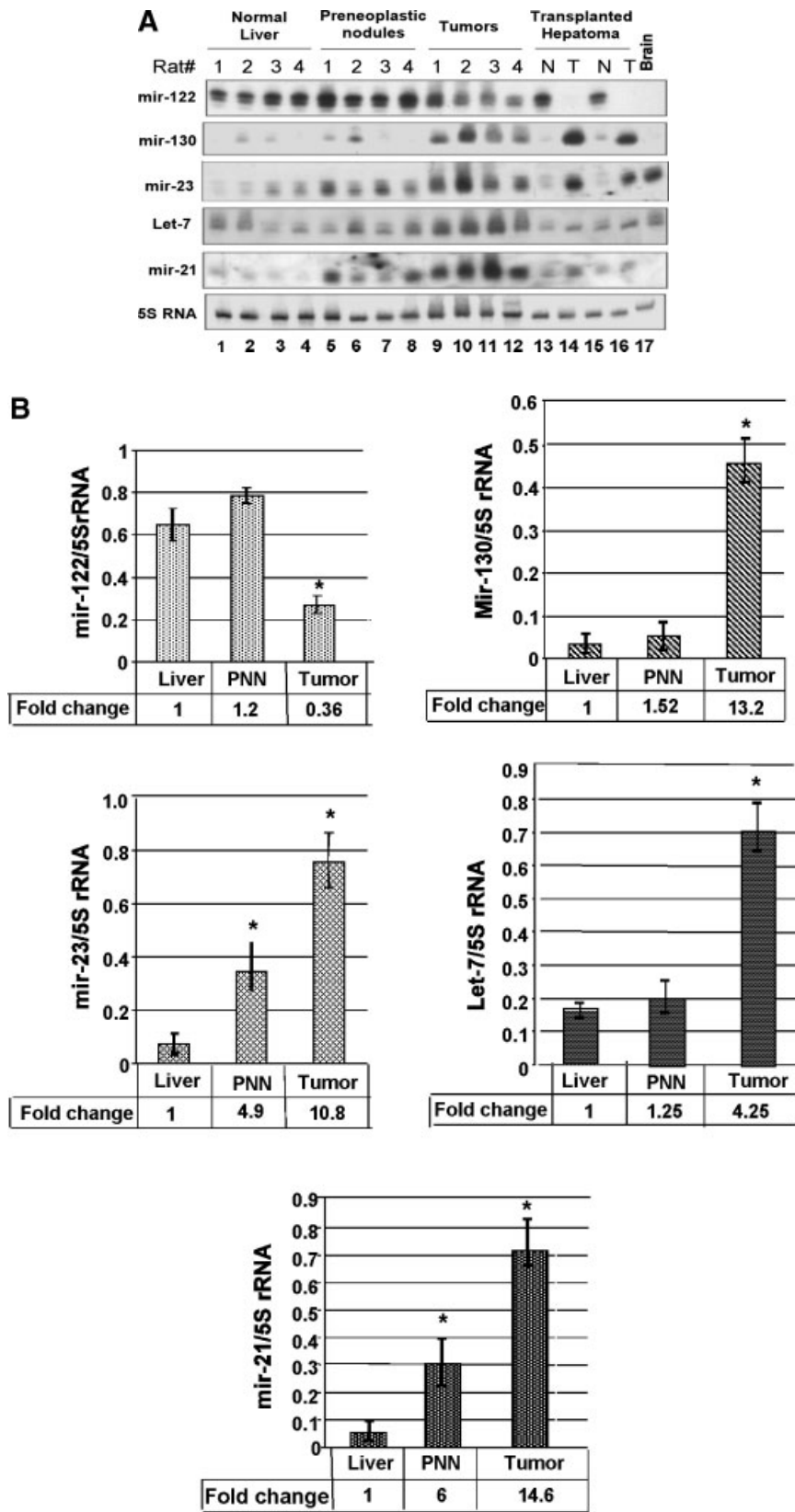


Fig. 2.

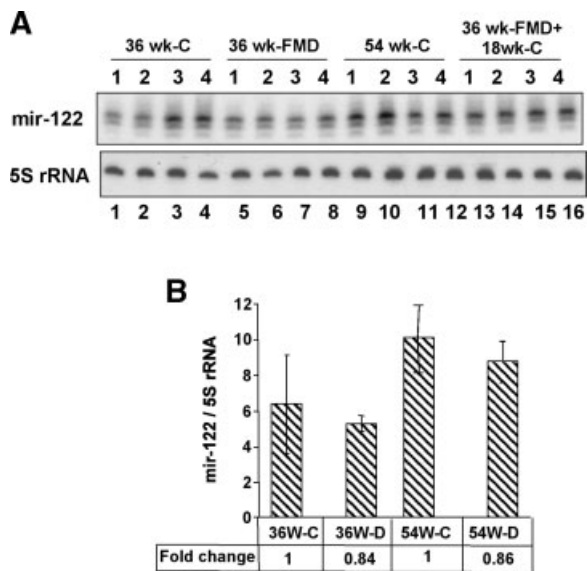


Fig. 3. A: Downregulation of miR-122 does not occur in the livers of rats switched to folate/methyl-adequate diet after 36 weeks on the FMD diet. The control animals were on adequate diet for 54 weeks whereas deficient animals were on FMD diet for the same time period. The third group was provided FMD diet for 36 weeks followed by adequate diet for another 18 weeks. Total RNA isolated from the livers was analyzed by Northern blotting. **B:** The quantitative representation of the data in (A). The results represent the average signal of each miR normalized to that of 5S rRNA \pm SD. *P*-value of ≤ 0.05 was considered significant. The ratio of miR-122 to 5S rRNA in the controls (36 weeks-C and 54 weeks-C) was assigned a value of 1.

folate and methyl-adequate diet after 36 weeks (Fig. 3A,B). Histological studies showed that none of the animals switched to folate/methyl-adequate diet after 36 weeks on the deficient diet developed hepatomas whereas animals continued with the FMD diet developed tumors after 54 weeks (data not shown). These results show that miR-122 level is specifically downregulated in HCCs.

Northern blot analysis also confirmed the microarray data (Table I) on the upregulation of some miRs, such as miR-21, miR-23, miR-130, and let-7 in the tumors (Fig. 2A,B). Among these, miR-130 and let-7 were upregulated only in tumors. On the contrary, miR-21 and -23, expressed at a low-level in the liver, were upregulated also in the PNNs (Fig. 2A,B). Their levels increased even further in the tumors. Upregulation of miR-21 and miR-23 in PNNs suggest their potential involvement in tumorigenesis. Like miR-122, miR-130, and miR-21 were not detectable in the brain.

Expressions of miR-122, miR-23, and miR-130 Are Also Deregulated in a Transplanted Rat Hepatoma

To find out whether hepatocarcinogenesis induced by other agents can cause deregulation of these miR genes, the levels of some of these miRs were measured in a transplanted rat hepatoma originally induced in rats with methylmethane sulfonate [Majumder et al., 2002]. The results showed that miR-122 level was high in the host livers but was not detectable in the tumors (Fig. 2A). As observed in the rat primary hepatomas, both miR-130 and miR-23 were significantly upregulated in the transplanted hepatoma whereas let-7 and miR-21 were not altered (Fig. 2A,B). Deregulation of these miRs (miR-122, -23, and -130) in the FMD diet as well as chemical carcinogen-induced HCCs suggested to us their potential role in hepatocarcinogenesis.

miR-122 Is Downregulated in Human Primary Hepatocellular Carcinomas

As a first step to understand the significance of differential expression of miRs in hepatocarcinogenesis, we extended the analysis of the liver-specific miR-122 to human primary HCCs. Since miR-122 is highly expressed in the liver it could be detected by Northern blot analysis using as little as 5 μ g of total RNA (Fig. 3A). Analysis of RNA from 20 human HCC samples showed that miR-122 was significantly downregulated in 50% of the tumors (10 out of 20) compared to non-malignant liver tissue from the same individuals (Fig. 4A,B). In some HCCs (sample #8, 11, 14, 17, and 19) miR-122 expression was not detectable whereas in others (#1, 3, 4, 7, and 9) it was significantly reduced. Among HCCs cell lines miR-122 was not detectable in human (HepG2, Hep3B) or mouse (Hepa) cells and expressed at a significantly low-level in human (H-7) [Chang et al., 2004] and rat (H4) cells compared to the liver (data not shown). These data demonstrate that miR-122 is downregulated in primary HCCs as well as hepatoma cell lines.

DISCUSSION

miRs first identified in *C. elegans*, play essential role in developmental timing [Ambros, 2004]. Recently, considerable attention has been focused on the expression of miRs in cancer because many of those miRs are located in

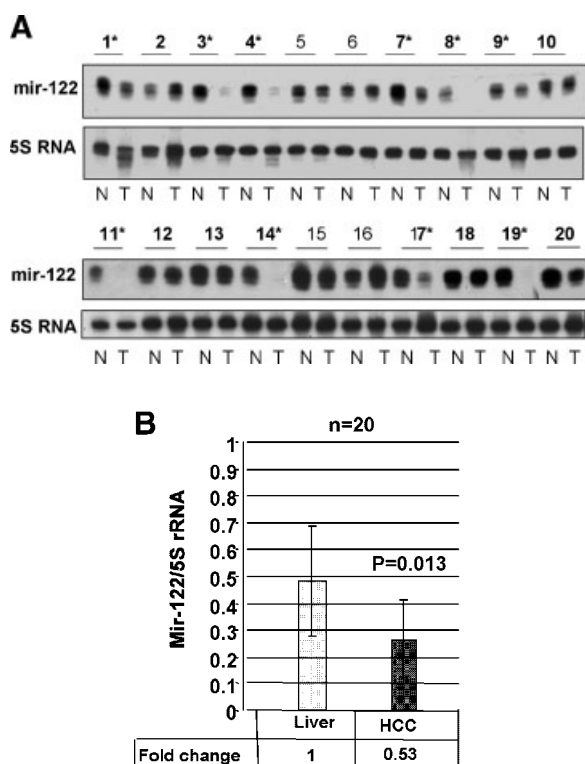


Fig. 4. Reduced expression of miR-122 in human primary HCCs. **A:** An aliquot (5 μ g) of total RNA from tumor (T) and matching normal (N) tissues was subjected to Northern blot analysis. Asterisks denote human primary HCCs in which miR-122 is downregulated. The signal of miR-122 normalized to that of 5S rRNA is presented below each sample. Asterisks indicate HCCs with significant decrease in miR-122. **B:** Quantitative analysis in all 20 samples showed that the decrease in miR-122 in HCCs was statistically significant.

fragile sites of chromosomes that are often translocated or deleted in human cancers and some of the miRNAs act as oncogenes or tumor suppressors [Volinia et al., 2006]. The present study, undertaken to identify miRNAs potentially involved in hepatocarcinogenesis induced by folate deficiency in a rat model, showed that miR expression was indeed deregulated.

The function of only a few of the miRNAs is known. miR-23 is involved in the retinoic acid-induced differentiation of NT2 cells [Kawasaki and Taira, 2003]. The antiapoptotic miR-21 is overexpressed in a variety of solid tumors [Volinia et al., 2006]. These miRNAs belong to the miR-17-92 cluster (that includes miR-17, -20, -93, and -106) on human chromosome 13 are upregulated by c-myc [He et al., 2005]. Among these miRNAs, miR-17-5p and miR-20a block translation of another c-myc target gene E2F1 involved in cell proliferation [O'Donnell et al., 2005]. Thus, c-myc tightly controls cell growth

by activating both E2F1 and miR-17-92 locus. This represents the first example of a mammalian transcription factor that regulates miR expression. Further, the miR-17-92 cluster facilitated c-myc-induced lymphomagenesis in a mouse model [He et al., 2005]. Together, these studies support an important role for this group of miRNAs in c-myc-mediated tumorigenesis. Interestingly, these miRNAs (miR-17, -20, -93, and -106) show similar expression pattern in rat HCCs and are clustered together (Fig. 1). c-myc is upregulated in rat HCC induced by FMD diet [Motiwala et al., 2003], which probably explains increased levels of some of the miRNAs in miR-17-92 locus in tumors (Tables I and II). Surprisingly, let-7 that negatively regulates RAS expression [Johnson et al., 2005], and is downregulated in human lung cancers [Takamizawa et al., 2004], is upregulated in rat HCCs.

miR-122 is a developmentally regulated liver-specific miR that could be detected as early as 12.5 days post-implantation and reaches a plateau immediately before birth [Chang et al., 2004]. Its level increases afterbirth at a much slower rate. This data suggest that miR-122 may play a critical role in liver development. Since it is specifically and abundantly expressed in the hepatocytes it may be involved in hepatocyte differentiation. miR-122 null mice will address its role, if any, in the liver development and tumorigenesis. Recent studies in mice using modified antisense miR-122 have shown that its depletion compromises the liver function and reduces cholesterol level by targeting expression of genes involved in cholesterol biosynthesis [Krutzfeldt et al., 2005]. It has also been shown that miR-122 modulates expression of Hepatitis C viral RNA by interacting with 5' non-coding region of the viral genome [Jopling et al., 2005]. Mutation of the miR-122-binding site does not affect the viral RNA translation or stability but facilitates replication of the viral RNA.

Our study demonstrates that miR-122 that constitutes 70% of the total hepatic miR [Chang et al., 2004] is downregulated in HCCs of both rodent and human origins. Analysis of the case report for some of the tumor samples did not reveal that downregulation of miR-122 in HCC is restricted to a specific etiology. It is critical to analyze a large number of HCC samples to determine whether reduced expression of miR-122 is correlated with specific etiology, ethnic

background or tumor grade that might help us show whether miR-122 can be used as a diagnostic marker.

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