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Down-Regulation of the microRNAs *miR-34a*, *miR-127*, and *miR-200b* in Rat Liver During Hepatocarcinogenesis Induced by a Methyl-Deficient Diet

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Altered expression of microRNAs (miRNAs) has been reported in diverse human cancers; however, the down-regulation or up-regulation of any particular miRNAs in cancer is not sufficient to address the role of these changes in carcinogenesis. In this study, using the rat model of liver carcinogenesis induced by a methyl-deficient diet, which is relevant to the hepatocarcinogenesis in humans associated with viral hepatitis C and B infections, alcohol exposure and metabolic liver diseases, we showed that the development of hepatocellular carcinoma (HCC) is characterized by prominent early changes in expression of miRNA genes, specifically by inhibition of expression of microRNAs *miR-34a*, *miR-127*, *miR-200b*, and *miR-16a* involved in the regulation of apoptosis, cell proliferation, cell-to-cell connection, and epithelial-mesenchymal transition. The mechanistic link between these alterations in miRNAs expression and the development of HCC was confirmed by the corresponding changes in the levels of E2F3, NOTCH1, BCL6, ZFHX1B, and BCL2 proteins targeted by these miRNAs. The significance of miRNAs expression dysregulation in respect to hepatocarcinogenesis was confirmed by the persistence of these miRNAs alterations in the livers of methyl-deficient rats re-fed a methyl-adequate diet. Altogether, the early occurrence of alterations in miRNAs expression and their persistence during the entire process of hepatocarcinogenesis indicate that the dysregulation of microRNAs expression may be an important contributing factor in the development of HCC.

Key words: microRNAs; p53; apoptosis; hepatocarcinogenesis

INTRODUCTION

Accumulated findings during recent years have established a critical role of microRNAs (miRNAs) in a large number of cellular processes [1,2]. miRNAs have been shown to be negative regulators of the expression of hundreds of genes involved in development, cell differentiation, metabolic regulation, cell proliferation, and apoptosis [3,4]. These processes, particularly enhanced cell proliferation and the dysregulation of cell death, are hallmark features of cancer cells, and are commonly altered during tumorigenesis [5,6]. Considering the regulatory role of miRNAs in controlling these pathways, it has been hypothesized that dysregulation of the miRNAome may have an intrinsic function in cancer. The first evidence indicating that miRNAs play an important role in cancer was reported by Calin et al. [7] who showed decreased expression of the *miR-15-a* and *miR-16-1* genes in human B cell chronic lymphocytic leukemia. Since then, a large amount of data have documented the profound effect of miRNAome deregulation in diverse human cancers [8,9], including liver cancer [10,11].

In cancer cells, miRNAs may function either as tumor-suppressors or oncogenes [12–14], of which the respective down- or up-regulation results in the establishment of highly tumor-specific miRNA fingerprints. However, the altered expression of any particular miRNAs in cancer, by itself, is not sufficient to address conclusively the role of these changes in tumorigenesis [13]; the altered expression may simply be a secondary consequence of malignant cell transformation reflecting the undifferentiated state of tumors. A recent report showing that global repression of miRNA maturation promoted cellular transformation provided the first experimental evidence of a causative role of miRNAs perturbation in carcinogenesis [15].

Additional supporting information may be found in the online version of this article.

Abbreviations: miRNA, microRNA; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR.

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Hepatocellular carcinoma (HCC) is one of the most common human cancers; it accounts for approximately 85% of all liver cancers and is showing an increased incidence throughout the world [16]. The development and progression of HCC in humans is a multi-step, long-term process, characterized by the progressive accumulation of genetic and epigenetic alterations associated with sequential evolution of morphologically distinct stages culminating in the formation of fully developed HCC [17]. In humans, most of the research on HCC is conducted on patients who have already developed the disease, which limits the scope of the investigation to tumor biology and does not allow extensive inquiry into the mechanisms of disease initiation and progression. By comparison, relevant rodent models of liver carcinogenesis provide a unique opportunity to understand the role of the etiologic factors and mechanisms of tumor development [18].

One of the most extensively studied models of HCC is the methyl-deficient model of liver carcinogenesis in rats. This model is unique because dietary omission of sources of methyl groups rather than xenobiotic addition leads to tumor formation [19]. In addition, the sequence of pathological and molecular events is remarkably similar to the development of human HCC associated with viral hepatitis B and C infections, alcohol exposure, and metabolic liver diseases [20].

We previously reported the substantial alterations in expression of the number of miRNAs in HCC induced by methyl deficiency in rats and also in human HCC [21], specifically the prominent down-regulation of liver-specific *miR-122a*. Recently this observation was confirmed with a larger set of human HCC samples [11]. Considering the involvement of miR-122a in the p53 pathway via *cyclin G1* gene targeting [11,22], a network frequently dysregulated during development of HCC [23,24], in the present study we investigated whether or not the development of HCC is associated with the alteration in the expression of miRNAs involved in the regulation of cell proliferation and apoptosis and the role of these miRNA alterations in the carcinogenic process.

MATERIALS AND METHODS

Animals, Diets, and Tissue Preparations

Male weaning Fisher 344 (F344) rats were obtained from the National Center for Toxicological Research (NCTR) breeding facility, housed 2 per cage in a temperature-controlled (24°C) room with a 12 h light/dark cycle, and given ad libitum access to water and NIH-31 pelleted diet (Purina Mills, Richmond, IN). At 4 wk of age, the rats (body weight 50 g) were allocated randomly to receive either a low methionine (0.18%) diet, lacking in choline and folic acid (Dyets, Inc., Bethlechem, PA), or a control diet supplemented with 0.4% methionine, 0.3% choline bitartrate, and 2 mg/kg folic acid. In addition, two groups of rats were maintained on the methyl-deficient diet for 9 and 18 wk, respectively, followed by re-feeding a methyl-adequate diet with a sufficient content of methionine, choline, and folic acid. Four rats per diet group and control group were sacrificed at 9, 18, 36, and 60 wk after diet initiation. The livers were excised, individual tumors were dissected, and all tissue samples were frozen immediately in liquid nitrogen, and stored at −80°C for subsequent analyses. Individual tumors were dissected from livers prior to freezing in liquid nitrogen. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the NCTR.

Quantitative Real-Time PCR (qRT-PCR) miRNAs Expression Analysis

Total RNA isolated from the liver tissue samples using TRI reagent (Ambion, Austin, TX) according to the manufacturer's instruction. To prevent genomic DNA contamination, all RNA samples were subjected to DNase I digestion. The quality of the total RNA was evaluated using Agilent 2100 Bionalyzer (Agilent Technologies, Palo Alto, CA). qRT-PCRs were performed by using SuperTaq Polymerase (Ambion) and a mirVana qRT-PCR miRNA Detection Kit (Ambion) following the manufacturer's instructions. Reactions contained mirVana qRT-PCR Primer Sets specific for miR-16a, miR-17-5p, miR-18, miR-19b, miR-20a, miR-21, miR-34a, miR-127, miR-181a, miR-200b, and let-7; rat 5S RNA served as the internal control. qRT-PCR was performed on an iCycler (BioRad, Hercules, CA). All reactions were run in triplicate. The threshold cycle (Ct) is defined as defined the fractional cycle number at which the fluorescence passes the threshold. The ΔCt was calculated by subtracting the Ct of 5S RNA from the each individual miRNA. Statistical significance ($P < 0.05$) was determined using an unpaired *t*-test by comparing the means of the methyl-deficient groups with those of the corresponding control groups. For data presentation, ΔΔCt values were calculated by subtracting the ΔCt of the age-matched normal liver sample from the ΔCt of age-matched corresponding methyl-deficient liver sample from which fold change in gene expression in rats fed a methyl-deficient diet relative to the age-matched control rats was determined [25]. The results presented as fold change of each miRNA in liver of rats fed methyl-deficient diet relative to control rats.

Western Immunoblotting

Liver tissue samples were prepared by homogenization of 50 mg of tissue in 500 μL of lysis buffer (50 mM Tris–HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM

PMSF; 1 μg/mL each aprotinin, leupeptin, pepstatin; 1 mM Na_3VO_4, 1 mM NaF), sonication, and incubation at 4°C for 30 min, followed by centrifugation at 10 000*g* at 4°C for 20 min. Equal amounts of proteins (50 μg) were separated by SDS–polyacrylamide electrophoresis in slab gels of 8 or 12% polyacrylamide, made in duplicate, and transferred to PVDF membranes (GE Healthcare Biosciences, Piscataway, NJ). Membranes were incubated with antibodies against BCL2 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), BCL6 (1:200, Santa Cruz Biotechnology), E2F3 (1:200, Santa Cruz Biotechnology), NOTCH1 (1:250; Abgent, San Diego, CA), ZFHX1B (1:1,000; Abnova, Taipei, Taiwan), c-MYC (1:200, Santa Cruz Biotechnology), and p53 (1:500; Cell Signaling Technology, Danvers, MA). Antibody binding was revealed by incubation with alkaline phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology). Chemifluorescence detection was performed with the ECF Substrate for Western Blotting (GE Healthcare) and measured directly by Storm Imaging System (Molecular Dynamics, Sunnyvale, CA). Images are representative of three independent immunoblots were normalized to β-actin and analyzed by ImageQuant software (Molecular Dynamics). All membranes were stained with Coomassie Blue to confirm equal protein loading.

Cell Culture

The nontumorigenic rat liver TRL1215 cell line was obtained from Dr. M.P. Waalkes (National Cancer Institute at National Institute of Environmental Health Sciences, Research Triangle Park, NC). Cells were cultured in William's E medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and 50 μg/mL penicillin–streptomycin as described previously [26].

Cell Transfection With Anti-miR-34a and Anti-miR-127

TRL1215 cells were seeded in six-well plates at a density of 1×10^5 cells/mL, and transfected with scrambled RNA oligonucleotide, 100 nM of anti-miR-34a, or 100 nM of anti-miR-127 (Ambion), in three independent replicates, using siPORT *NeoFX* transfection agent (Ambion) in accordance with the manufacturer's protocol. At 72 h post-transfection, adherent cells were harvested by mild trypsinization, re-seeded, and transfection was repeated. The transfection procedure was consequently repeated five times. The viability of cells was monitored by MTT test after each transfection. Seventy-two hours after last transfection, the levels of endogenous miR-34a and miR-127 were assessed by qRT-PCR as described above.

Apoptosis Assay

Twenty-four hours after the last transfection, control, anti-miR-34a- or anti-miR-127-transfected TRL1215 cells were gamma-irradiated at a final dose of 5 Gy. Apoptosis was determined by caspase activation 24 h after irradiation using Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega, Madison, WI) according to the manufacturer's protocol. The protein concentration was determined by Bradford assay (Pierce, Rockford, IL), and caspase activities for all samples were normalized to that of an equal protein amount. The experiments were repeated twice in triplicate.

qRT-PCR Array Analysis of the p53 Signaling Pathway Gene Expression

Expression of the genes involved in the p53 signaling network in the TRL1215 cells transfected with anti-miR-34a or anti-miR-127 was determined using RT^2Profiler PCR Array System for p53 signaling pathway (SuperArray, Frederick, MD) according to the manufacturer's protocol.

Statistical Analysis

Results are presented as mean ± SD. Statistical analyses were conducted by two-way ANOVA, with pair-wise comparisons being conducted by Student–Newman–Keuls test.

RESULTS

Expression of miRNAs in HCC Induced by Methyl Deficiency in Rats

Development of liver tumors induced by methyl deficiency is characterized by the progressive sequential evolution of well-defined morphologically distinct stages. The initial morphological changes (within first 3 wk) are associated with steatosis and increased apoptosis that is coupled with compensatory liver regeneration [27]. By 9 wk, the liver tissue is characterized by formation of small glutathione-S-transferase (GSTπ)-positive foci [27,28]. The accumulated evidence suggests that the presence of enzyme-altered foci in the liver is a sensitive marker for initiated cells representing precursor lesions, which are causally related to the carcinogenic process in the liver. Fibrosis, accompanied by progressive expansion of GSTπ-positive foci, develops by 18 wk. At this time point, large GSTπ-positive foci are detected in all liver samples. By 36 wk, fibrosis progresses to cirrhosis and is characterized by disruption of liver architecture and formation of preneoplastic nodules in the livers [28,29]. After 54 wk of exposure to a methyl-deficient diet, HCC develops with a 100% incidence [19,20,30].

Table 1 shows that rat HCC induced by methyl deficiency is characterized by aberrant expression of miRNAs that targets known tumor suppressors and oncogenes involved in the maintenance of the balance between apoptosis and cell proliferation, specifically by profound down-regulation of the tumor suppressors *miR-34a*, *miR-16a*, *miR-127*,

Table 1. miRNA Expression in HCC Induced by Methyl Deficiency in Rats

miRNA	Fold change ($2^{-\Delta\Delta Ct}$)[a]		Confirmed and predicted miRNA targets
	Control	HCC	
rno-miR-34a	1.02 ± 0.09	0.42 ± 0.05[a]	E2F3 [31,32], NOTCH1[33]
rno-miR-16a	1.08 ± 0.04	0.29 ± 0.05[a]	BCL2 [34]
rno-miR-127	0.97 ± 0.06	0.18 ± 0.03[a]	BCL6 [35]
rno-miR-181a	1.08 ± 0.09	0.23 ± 0.06[a]	TCL1 [36]
rno-miR-21	1.02 ± 0.13	0.78 ± 0.16	PTEN [39], PDCD4 [40]
rno-miR-17-5p	1.04 ± 0.02	0.85 ± 0.10	E2F1 [38]
rno-miR-19b	1.00 ± 0.02	0.45 ± 0.13	E2F1 [38]
rno-miR-20a	1.05 ± 0.02	0.65 ± 0.04[a]	E2F1 [38]
let-7	1.03 ± 0.10	0.96 ± 0.07	Multiple genes [41]
rno-miR-200a	1.06 ± 0.13	0.73 ± 0.18	BAP1[b]
rno-miR-200b	1.04 ± 0.05	0.42 ± 0.02[a]	ZFHX1B [37]
rno-miR-200c	1.04 ± 0.23	0.81 ± 0.28	TCF8 [44]

Data presented as average fold change of each miRNA normalized to that of 5S RNA in HCC compared to liver of control rats. The level of each microRNA expression was measured using the $2^{-\Delta\Delta Ct}$ method [25]. These values represent the mean $\pm$ SD of four biological replicates.
[a]Significantly different ($P < 0.05$) from control.
[b]Predicted targets by PicTar, and TargetScan 4.1.

miR-181a, and *miR-200b* that regulate E2F3 (*miR-34a*) [31,32], NOTCH1 (*miR-34a*) [33], BCL2 (*miR-16a*) [34], BCL6 (*miR-127*) [35], TCL1 (*miR-181a*) [36], and ZFHX1B (*miR-200b*) (ZEB2, SIP1, SMADIP1) [37]. On the other hand, the expression of the components of the oncogenic *miR-17-92* cluster (*miR-17-5p*, *miR-19b*, and *miR-20a*) that targets the E2F1 transcription factor and c-MYC [38] decreased to a lesser extent. In contrast, the expression of the *miR-21* that regulates PTEN and PDCD4 tumor-suppressors [39,40] and *let-7* that regulates multiple genes involved in cell proliferation pathways [41] did not change (Table 1).

Expression of miRNAs in the Livers During Hepatocarcinogenesis Induced by Methyl Deficiency in Rats

In order to evaluate whether or not alterations in miRNAs play a significant role in the development of HCC or are simply a consequence of the transformed state of cancer cells, we measured the expression of miRNAs, using qRT-PCR, at different stages of hepatocarcinogenesis. In the livers of rats fed the methyl-deficient diet, *miR-34a*, *miR-127*, and *miR-200b* became significantly down-regulated after 9 and 18 wk on the diet (Fig. 1). At that time, the expression of these miRNAs was ~80–90% lower than in the age-matched control rats. A similar pattern of changes was observed for *miR-16a* expression; however, the inhibition of *miR-16a* expression occurred to a lesser extent than the alterations of *miR-34a*, *miR-127*, and *miR-200b*. At later times (36 wk), the expression of *miR-34a*, *miR-127*, *miR-200b*, and *miR-16a* increased slightly compared to the 9 and 18 wk values, which may be explained by intensive fibrogenesis, hepatocyte regeneration, and oval cell proliferation that occurred at these time in the livers of methyl-deficient rats. However, the expression of these miRNAs still remained ~40–60% lower than the control values.

In contrast, expression of miRNAs, components of oncogenic *miR-17-92* polycistron, such as *miR-17-5p* (Fig. 1) and *miR-20a* (data not shown), did not change in the livers of rats maintained on the methyl-deficient diet, while expression of oncogenic miRNA *miR-19b* showed a slight, but statistically significant, decrease only after 36 wk of deficiency (Fig. 1). The expression of *let-7*, *miR-21*, *miR-181a*, *miR-200a*, and *miR-200c* was also not altered (data not shown).

Protein Expression of miRNA Targets in the Livers During Hepatocarcinogenesis Induced by Methyl Deficiency in Rats

To establish the mechanistic meaning of miRNAs expression dysregulation in hepatocarcinogenesis, we determined the protein levels of the experimentally confirmed targets of these differentially expressed miRNAs. Western blot analysis showed increased protein levels of E2F3 and NOTCH1 (miR-34a), BCL2 (miR-16a), BCL6 (miR-127), and ZFHX1B (miR-200b) [31–35,37] in the livers of rats fed a methyl-deficient diet, while the protein levels of c-MYC, directly activated and targeted by miRNAs from oncogenic *miR-17-92* cluster [38], did not change (Fig. 2).

To prove the significance of miRNAs expression dysregulation in hepatocarcinogenesis, we evaluated the evolution of miRNAs alterations in the livers of methyl-deficient rats re-fed a methyl-adequate diet.

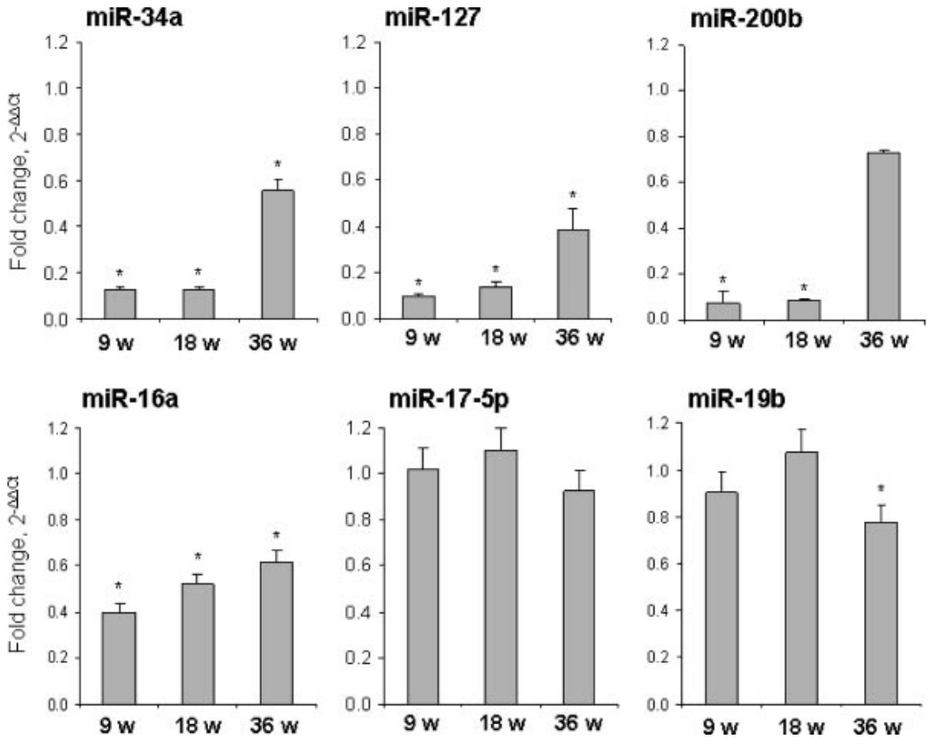

Figure 1. qRT-PCR of differentially expressed miRNA genes in the livers of control rats and rats fed methyl-deficient diet. Expression changes of *miR-34a*, *miR-127*, *miR-200b*, *miR-16a*, *miR-17-5p*, and *miR-19b*, in the livers during rat hepatocarcinogenesis induced by methyl deficiency. The miRNA expression data presented as average fold change of each miRNA normalized to that of 5S RNA in liver of methyl-deficient rats compared to control rats. The level of each microRNA expression was measured using the $2^{-\Delta\Delta Ct}$ method [25]. The results presented as fold change of each miRNA in liver of rats fed methyl-deficient diet relative to control rats. These values represent the mean of four biological replicates. * Significantly different from age-matched control rats ($P < 0.05$).

Figure 3 demonstrates that re-feeding rats maintained on the methyl-deficient diet for 9 wk the methyl-adequate diet led to a normalization of expression of *miR-34a*, *miR-200*b, and *miR-16a*, but failed to restore the expression of *miR-127*. In rats exposed to methyl deficiency for longer time ($\geq$18 wk), expression of these miRNAs, particularly *miR-34a*, *miR-127*, and *miR-200b*, remained down-regulated to the end of the experiment despite feeding the animals a methyl-adequate diet (Fig. 3). These changes were accompanied by the high-incidence of hyperplastic nodules and by the persistence of GSTπ-positive foci in the livers of re-fed rats [42].

In order to provide evidence that down-regulation of miR-34a and miR-127 diminishes the cellular apoptotic program, we transfected liver TRL1215 cells with anti-miR-34a or anti-miR-127 and induced apoptosis using gamma-radiation, a well-known genotoxic inducer of programmed cell death. The results of our in vitro experiments demonstrated that down-regulation of *miR-34a* and *miR-127* to a similar extent as in preneoplastic livers inhibits apoptosis in liver TRL1215 cells, which was evidenced by a decreased expression of genes involved in the p53-pathway and reduced apoptotic response in irradiated cells (Fig. 4 and Supplementary Table 1). Additionally, these results indicate a mechanistic link between the down-regulation of miR-34a and miR-127 and the dysregulated apoptosis frequently observed during liver carcinogenesis. This suggestion was further supported by evidence of down-regulation of *p53* gene expression [43], decreased levels of p53 protein, and diminished caspase activity in HCC induced by methyl-deficiency (Fig. 5).

DISCUSSION

The results of the study show that rat hepatocarcinogenesis induced by a methyl-deficient diet is characterized by a prominent inhibition of expression of miRNA genes responsible for the maintenance of balance between cell proliferation and apoptosis. More importantly, the early occurrence of these alterations and their persistence during the entire process of hepatocarcinogenesis demonstrated clearly that aberrant miRNAs expression may be an important contributing factor in the development of HCC.

Accumulated findings over recent years have established a crucial role of miRNAs in cancer [8,9,12–14], leading to a suggestion that the dysregulation of miRNAome may contribute to the

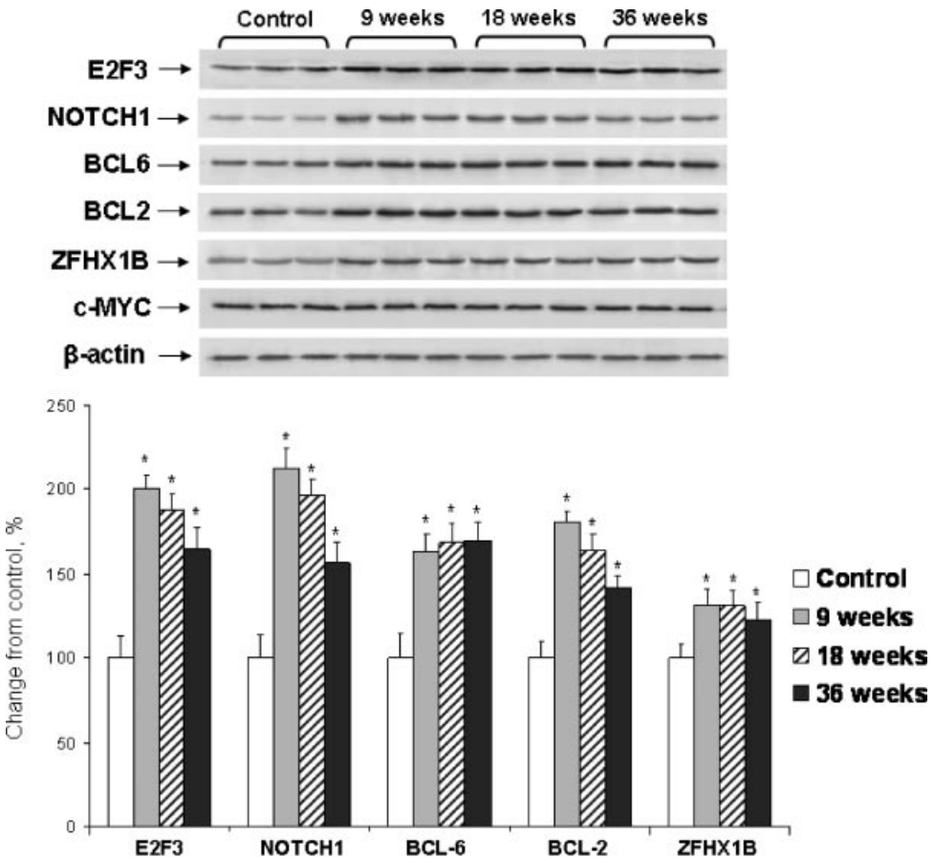

Figure 2. Western blot analysis of the E2F3, NOTCH1, BCL6, BCL2, ZFHX1B, and c-MYC proteins in the livers of control and rats and rats fed methyl-deficient diet. (A) Liver tissue lysates were separated by SDS–PAGE and subjected to the immunoblotting using specific antibodies against the E2F3, NOTCH1, BCL6, BCL2, ZFHX1B, and c-MYC proteins. Equal sample loading was confirmed by immunostaining against β-actin. These results were reproduced in two independent experiments. (B) Quantitative evaluation of the E2F3, NOTCH1, BCL6, BCL2, ZFHX1B, and c-MYC proteins in the livers of control rats and rats fed methyl deficient diet. Protein levels are presented as relative to age-matched control rats after normalization to β-actin ($n = 4$, mean ± SD). * Significantly different from age-matched control rats ($P < 0.05$).

initiation and progression of cancer. However, most of the cancer-miRNA-related studies are based on expression analyses of tumor cells in comparison with normal cells. The altered expression of any particular miRNAs in cancer cells by itself is not sufficient to address conclusively the role of these changes in carcinogenesis. To provide evidence that miRNAs dysregulation plays an important role in cancer development, it is necessary to demonstrate that: (a) altered miRNA expression occurs at a considerable frequency in early stages of carcinogenesis; (b) changes that occur at preneoplastic stages are also present during later stages of cancer; (c) additional changes are acquired during tumor progression; and (d) a mechanistic link exists between these changes and cancer development. In this respect, alterations in the expression of tumor suppressor miRNAs *miR-34a*, *miR-127*, *miR-16a*, and *miR-200b* during development of HCC in rats induced by a methyl-deficient diet correspond to the above parameters. This was evidenced particularly by the early occurrence and persistent down-regulation of these miRNAs during hepatocarcinogenesis. In contrast, inhibition of expression of the *miR-181a* and *miR-20a* occurred in fully developed HCC only.

To obtain information about a mechanistic link between the alterations in miRNAs expression and the development of HCC induced by methyl-deficiency, we studied the association of *miR-34a*, *miR-16a*, *miR-127*, and *miR-200b* expression. These miRNAs displayed the most prominent and persistent changes during hepatocarcinogenesis and in the levels of their targets—transcription factor E2F3, NOTCH1, BCL2, BCL6, and ZFHX1B proteins, respectively. The highest levels of E2F3, NOTCH1, BCL2, BCL6, and ZFHX1B proteins in the livers of methyl-deficient rats were detected at 9 and 18 wk of deficiency and were inversely related to the expression of corresponding miRNAs (Figs. 1 and 2). More importantly, these alterations in expression of miRNAs and their targets were associated with the morphological changes that occurred at that time in the liver tissues of methyl-deficient rats. Specifically,

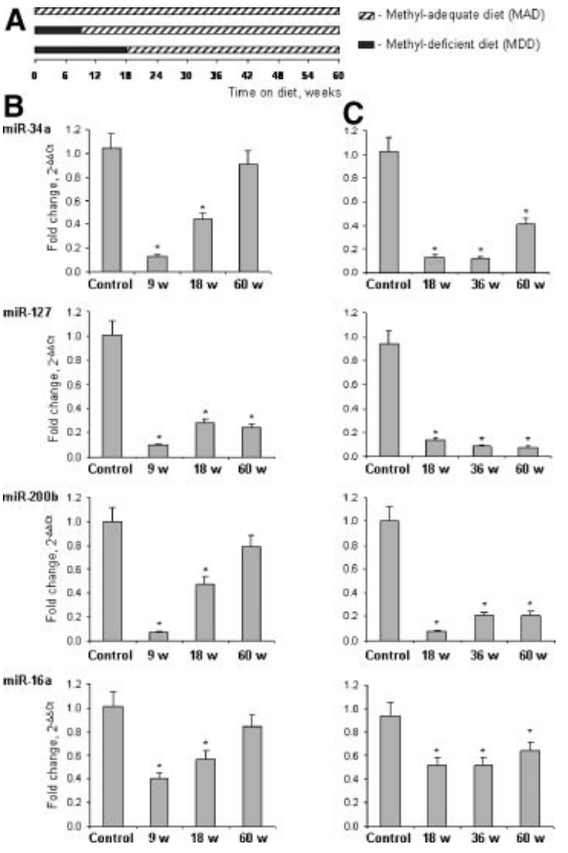

Figure 3. Expression changes of *miR-34a*, *miR-127*, *miR-200b*, and *miR-16a* genes in the livers of methyl-deficient rats re-fed methyl-adequate diet. (A) Diagram of the feeding protocol. Male F344 rats were maintained on a methyl-deficient diet for 9 and 18 wk followed by re-feeding a methyl-adequate diet. (B,C) qRT-PCR of *miR-34a*, *miR-127*, *miR-200b*, and *miR-16a* genes in the livers of F344 maintained on a methyl-deficient diet for 9 wk (B) and 18 wk (C) followed by re-feeding a methyl-adequate diet. Four rats per diet group and control group were sacrificed at 9 and 18 wk of methyl-deficiency and after 9 wk of re-feeding (at 18 wk of the study), 18 wk of re-feeding (at 36 wk of the study), and at the end of the experiment (at 60 wk of the study). The results presented as fold change of each miRNA relative to control rats. These values represent the mean of four biological replicates. * Significantly different from age-matched control rats ($P < 0.05$).

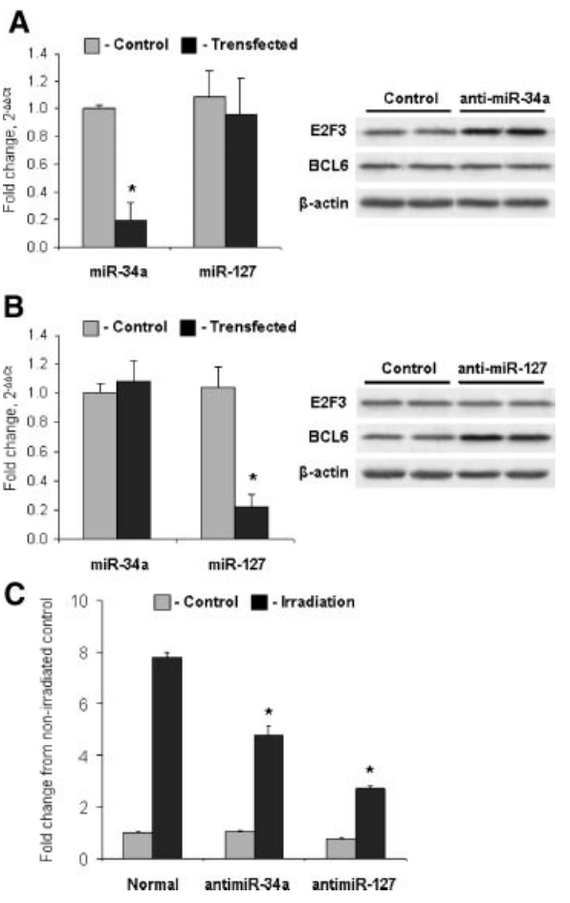

Figure 4. Inhibition of endogenous miR-34a or miR-127 compromises apoptosis in the rat liver TRL1215 cells. The rat liver TRL1215 cells were transfected with scrambled RNA oligonucleotides (control), 100 nM of anti-miR-34a, or 100 nM of anti-miR-127. Transfection with anti-miR-34a or with anti-miR-127 resulted in reduction of the endogenous levels of miR-34a (A) or miR-127 (B), and in increase in the protein levels of E2F3 (A) or BCL6 (B), respectively. Twenty-four hours after last transfection, TRL1215 cells were exposed to gamma irradiation (5 Gy) and after additional 24 h activation of caspase activity, indicative of apoptosis, was determined (C). The decrease in caspase activation in gamma-irradiated TRL1215 cells transfected with anti-miR-34a or anti-miR-127 compared to control cells is indicative of the reduction of apoptotic response. * Significantly different from control TRL1215 cells transfected with scrambled RNA oligonucleotide ($P < 0.05$). The experiments were repeated twice in triplicate.

the up-regulation of E2F3, NOTCH1, BCL2, and BCL6 proteins were accompanied by the imbalance between apoptosis and cell proliferation [27,28], and progressive liver fibronegesis [27,29] was accompanied by increased levels of ZFHX1B protein, a known inducer of epithelial-mesenchymal transition [45]. The pattern of changes in expression of miRNAs and their targets during this time corresponded to the results of our previous study demonstrating that crucial biological and biochemical changes associated with hepatocarcinogenesis occurred specifically between 9 and 18 wk of methyl deprivation [42].

Recent findings demonstrate a solid connection between the miR-34 family of miRNAs and p53 tumor suppressor network in the regulation of apoptosis [46,47], which is frequently dysregulated during hepatocarcinogenesis. In view of this, the down-regulation of *miR-34a* expression in the livers of methyl-deficient rats may contribute to a carcinogenic process by inhibition of the p53-pathway and compromising the cellular apoptotic program.

The reports by Welch et al. [31] and Tazawa et al. [32] have demonstrated that miR-34a directly targets the E2F3, a potent transcriptional inducer of the cell

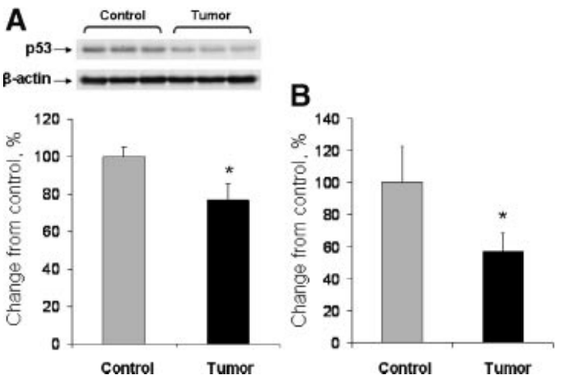

Figure 5. Apoptosis in hepatocellular carcinomas induced by methyl-deficiency. (A) Western blot analysis of the p53 protein in the livers of control rats and in hepatocellular carcinomas induced by a methyl-deficient diet. (B) Caspase-3/7 activity in the livers of control rats and in hepatocellular carcinomas induced by a methyl-deficient diet. * Significantly different from age-matched control rats ($P < 0.05$). These values represent the mean of four biological replicates.

cycle progression. A considerable body of evidence has revealed an essential role of the E2F family of transcription factors in the control of cellular proliferation by regulating the transcription of genes involved in cell cycle progression [48,49], especially a critical role of the E2F3 in the control of a p53-dependent checkpoint [41–51]. E2F3 acts as a classic activator of cell cycle progression; when over-expressed E2F3 is sufficient, by itself, to induce cellular proliferation [48] and may lead to tumor development [51]. The oncogenic property of E2F3 over-expression, in addition to its function as an inducer of cell proliferation, may also be due to its ability to repress the p53 pathway [49,50]. In this respect, down-regulation of *miR-34a* expression and consequent up-regulation of E2F3 may lead to malignant cell transformation via reduction of cellular apoptotic program by inhibition of p53-network. Furthermore, the up-regulation of NOTCH1 protein, another miR-34a target, BCL6 and BCL2 oncoproteins, may further disrupt the balance between cell proliferation and apoptosis, leading to neoplastic cell transformation by inhibiting the p53 activation [52,53]. This suggestion is supported by recent evidence showing the down-regulation of the miR-34a in the human HCC [54,55].

The early down-regulation of *miR-200b* and consequent up-regulation of the ZFHX1B, a strong inhibitor of E-cadherin and inducer of epithelial-mesenchymal transition, is an important finding of the study. Additionally, considering the fact that any alterations that persist after removal of the carcinogen are regarded as preneoplastic or neoplastic in nature [56], the stable down-regulation of *miR-34a*, *miR-127*, and *miR-200b* in the livers of methyl-deficient rats re-fed a methyl-adequate diet indicated the significance of miRNAs expression dysregulation in respect to hepatocarcinogenesis.

The results of our study provide evidence for the significance of the dysregulation of miRNAs expression in hepatocarcinogenesis. More importantly, the early occurrence of these miRNA alterations and their persistence during hepatocarcinogenesis demonstrated clearly that aberrant miRNAs expression may be an important causative factor in the development of HCC.

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