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.

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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-α 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 miRNA 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., Bethlehem, 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 instructions. 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
TLR1215 cells were seeded in six-well plates at a density of 1 x 10⁵ 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 the 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 TLR1215 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 TLR1215 cells transfected with anti-miR-34a or anti-miR-127 was determined using RT² Profiler 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 (GSTγ)-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 GSTγ-positive foci, develops by 18 wk. At this time point, large GSTγ-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,
miR-34a, and miR-200b that regulate E2F3 (miR-34a) [31,32], NOTCH1 (miR-34a) [33], BCL2 (miR-16a) [34], BCL6 (miR-127) [35], TCF8 (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 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-127, miR-200b, and miR-16a, but failed to restore the expression of miR-127. In rats exposed to methyl deficiency for longer time (≥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 GSTp-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
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, 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,
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 fibrogenesis [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
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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REFERENCES

1. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 2004;116:281–297.
2. Sevignani C, Calin GA, Siracusa LD, Croce CM. Mammalian microRNAs: A small world for fine-tuning gene expression. Mammm Genome 2006;17:189–202.
3. Bushati N, Cohen SM. microRNAs functions. Annu Rev Cell Dev Biol 2007;23:175–205.
4. Hwang H-W, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer 2006;94:776–780.
5. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
6. Evan GI, Voussen KH. Proliferation, cell cycle and apoptosis in cancer. Nature 2001;411:342–348.
7. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of microRNA genes miR15 and miR16 at 13q14 in chronic lymphatic leukemia. Proc Nat Acad Sci USA 2002;99:15524–15529.
8. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834–838.
9. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857–866.
10. Murakami Y, Yasuda T, Saigo K, et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 2006;25:2537–2545.
11. Gramantieri L, Ferracin M, Fornari F, 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.
12. Hammond SM. MicroRNAs and oncogenes. Curr Opin Genet Dev 2006;16:4–9.
13. Kent OA, Mendell JT. A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. Oncogene 2006;25:6188–6196.
14. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol 2007;302:1–12.
15. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet 2007;39:673–677.
16. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: From genes to environment. Nat Rev Cancer 2006;6:674–687.
17. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002;31:339–346.
18. Lee JS, Chu JS, Mikaelyan A, et al. Application of comparative functional genomics to identify best-fit mouse model to study human cancer. Nat Genet 2004;36:1306–1311.

19. Nakae D. Endogenous liver carcinogenesis in the rat. Pathol Int 1999;49:1028–1042.

20. Powell CL, Kosyk O, Bradford BU, et al. Temporal correlation of pathology and DNA damage with gene expression in a choline-deficient model of rat liver injury. Hepatology 2005;42:1137–1147.

21. Kutay H, Bai S, Datta J, et al. Downregulation of mir-122 in the rodent and human hepatocellular carcinomas. J Cell Biochem 2006;99:671–678.

22. Kimura SH, Nojima H. Cyclin G1 associates with MDM2 and inhibits tumour suppressor p53. Proc Natl Acad Sci USA 1997;94:10907–10912.

23. Hussain SP, Schwank J, Staib F, Wang XW, Harris CC. TP53 promoters CpG-binding protein, MeCP2, in rat liver. Carcinogenesis 1997;18:287–293.

24. Pitot HC. Advancements in hepatocarcinogenesis. Annu Rev Pathol 2007;2:1–29.

25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔct method. Methods 2001;25:402–408.

26. Zhao CQ, Young MR, Diwan BA, Coogan TP, Waalkes MP. Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. Proc Natl Acad Sci USA 1997;94:10907–10912.

27. James SJ, Miller BJ, Basnakian AG, Pogribny IP, Pogribna M. Alterations in hepatic p53 expression of pathology and DNA damage with gene expression in a choline-deficient model of rat liver injury. Hepatology 2005;42:1137–1147.

28. Pogribny IP, Muskhelishvili L, Miller BJ, James SJ. Presence and consequence of uracil in preneoplastic DNA from folate/ methyl-deficient rats. Carcinogenesis 1997;18:2071–2076.

29. Pogribny IP, Muskhelishvili L, Miller BJ, James SJ. Methylation deficiency causes reduction of the methyl-CpG binding protein, MeCP2, in rat liver. Carcinogenesis 2003;24:1935–1940.

30. Pogribny IP, James SJ. De novo methylation of the p16INK4A gene in early preneoplastic liver and tumors induced by folate/methyl deficiency in rats. Cancer Lett 2002;187:69–75.

31. Welch C, Chen Y, Stallings RL. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. Oncogene 2007;26:5017–5022.

32. Tazawa H, Tsuichaya N, Iziyama M, Nakagama H. Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. Proc Natl Acad Sci USA 2007;104:15472–15477.

33. Fukuda Y, Kawasaki H, Taira K. Exploration of human miRNA target genes in neuronal differentiation. Nucleic Acid Res 2005;49:341–342.

34. Cimmino A, Calin GA, Fabbrri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA 2005;102:13944–13949.

35. Saito Y, Liang G, Egger G, et al. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 2006;9:435–443.

36. Pekarsky Y, Santanam U, Cimmino A, et al. Tcf1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. Cancer Res 2006;66:11590–11593.

37. Christoffersen NR, Silahtaroglu A, Orom UA, Kauppinen S, Lund AH. miR-200b mediates post-transcriptional repression of Zfhx1B. RNA 2007;13:1172–1178.

38. O’Donnell KA, Wentzel EA, Zeller KJ, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 2005;435:839–843.

39. 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.

40. Asangani IA, Rashed SA, Nikolova DA, et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 2008;27:2128–2136.

41. Johnson CD, Esquela-Kerscher A, Stefani G, et al. The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res 2007;67:7713–7722.

42. Pogribny IP, Ross SA, Wise C, et al. Irreversible global DNA hypomethylation as a key step in hepatocarcinogenesis induced by dietary methyl deficiency. Mut Res 2006;593:80–87.

43. Pogribny IP, Miller BJ, James SJ. Alterations in hepatic p53 gene methylation patterns during tumor progression with folate/ methyl deficiency in the rat. Cancer Lett 1997;115:31–38.

44. Horst HU, Carlson JA, Spivack SD, Brock GJ. Overexpression of microRNA has-miR-200c leads to reduced expression of transcription factor b and increased expression of E-cadherin. Cancer Res 2007;67:7972–7976.

45. Vanderwalle C, Comijn J, De Craene B, et al. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions. Nucl Acid Res 2005;33:6566–6578.

46. Pogribny IP, James SJ, Miller BJ, et al. A microRNA component of the p53 tumour suppressor network. Nature 2007;447:1130–1135.

47. Heremeka H. p53 enters the microRNA world. Cancer Cell 2007;12:414–418.

48. Wuu L, Timmers C, Maiti B, et al. The E2F-1 transcription factors are essential for cellular proliferation. Nature 2001;414:457–462.

49. Sharma N, Timmers C, Trikha P, Saavedra HL, Obery A, Leone G. Control of the p53-p21Cip1 axis by E2f1, E2f2, and E2f3 is essential for G1/S progression and cellular transformation. J Biol Chem 2006;281:36124–36131.

50. Gissend B. E2F3—a novel repressor of the ARF/p53 pathway. Dev Cell 2004;6:742–743.

51. Paulson OA, MacArthur MJ, Johnson DG. E2F3a stimulates proliferation, p53-independent apoptosis and carcinogenesis in a transgenic mouse model. Cell Cycle 2006;5:184–190.

52. Kim SB, Chae GW, Lee J, Park J, Tak H, Chung HJ, Park TG, Ahn JK, Joe CO. Activated Notch1 interacts with p53 to inhibit its phosphorylation and transactivation. Cell Death Differ 2007;14:981–991.

53. Phan RT, Dalla-Favera R. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. Nature 2004;432:635–639.

54. Jiang J, Gusev Y, Aderca I, et al. Association of MicroRNA expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. Clin Cancer Res 2008;14:419–427.

55. Budhu A, Jia HL, Forges M, et al. Identification of metastasis related microRNAs in hepatocellular carcinoma. Hepatology 2008;47:987–907.

56. Dragom VR, Pitot HC. The role of the stages on initiation and promotion in phenotypic diversity during hepatocarcinogenesis in the rat. Carcinogenesis 1992;13:739–750.