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Dietary Methyl Deficiency, microRNA Expression and Susceptibility to Liver Carcinogenesis

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MicroRNAs (miRNAs) are small 21–25 nucleotide-long non-coding RNAs that have emerged as key negative post-transcriptional regulators of gene expression [1, 2]. Currently there are more than 700 mammalian miRNAs that can potentially target up to one-third of protein-coding human genes [1] involved in diverse physiological and pathological processes, including cancer [3, 4]. Indeed, aberrant levels of miRNAs have been reported in all major human malignancies [5, 6]. In tumors, altered expression of miRNAs has been demonstrated to inhibit tumor suppressor genes or inappropriately activate oncogenes and has been associated with every aspect of tumor biology, including tumor progression, invasiveness, metastasis, and acquisition of resistance by malignant cells to chemotherapeutic agents [3, 4, 7, 8]. These observations lead to the suggestion that aberrant expression of miRNAs may contribute to tumorigenesis [9]. However, most of the tumor-miRNA-related studies are based on expression analysis of miRNAs in tumors in comparison with corresponding adjacent normal tissues [4–6]. The altered expression of any given miRNA in neoplastic cells is not sufficient to address conclusively the role of these changes in tumorigenesis [10]. Additionally, despite the established biological significance of miRNA dysregulation in neoplastic cells, there is a lack of knowledge on the role of miRNAs during early stages of tumor development, especially if variations in the expression of specific miRNAs are associated with differences in the susceptibility to tumorigenesis.

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In light of these considerations, the goals of this study were to: (1) define the role of miRNA dysregulation in early stages of liver carcinogenesis, and (2) determine how these alterations in miRNA expression may be mechanistically linked to the pathogenesis of liver cancer induced by dietary methyl deficiency.

Materials and Methods

Animals, Diets and Experimental Design

Male C57BL/6J and DBA/2J mice (Jackson Laboratory, Bar Harbor, Me., USA) were housed in sterilized cages in a temperature-controlled room (24°C) with a 12-hour light/dark cycle, and given ad libitum access to purified water and NIH-31 pelleted diet (Purina Mills, Richmond, Ind., USA). At 8 weeks of age, the mice from each strain were allocated randomly into 2 groups, 1 control and 1 experimental. The mice in the experimental group were maintained on a low methionine (0.18%) diet, lacking in choline and folic acid (Dyets Inc, Bethlehem, Pa., USA) for 12 weeks. The mice in the control group received a diet supplemented with 0.4% methionine, 0.3% choline bitartrate and 2 mg/kg folic acid. Diets were stored at 4°C and given ad libitum, with twice a week replacement. Five experimental and 5 control mice were sacrificed at 12 weeks after diet initiation. The livers were excised, frozen immediately in liquid nitrogen, and stored at –80°C for subsequent analyses. All animal experimental procedures were carried out in accordance with the animal study protocol approved by the National Center for Toxicological Research Animal Care and Use Committee.

RNA Extraction and miRNA Microarray Expression Analysis

Total RNA was extracted from the liver tissue using miRNAeasy Mini Kit (Qiagen, Valencia, Calif, USA) according to the manufacturer's instructions. The miRNA microarray analysis was performed by LC Sciences (Houston, Tex., USA), as reported previously in detail [11].

miRNA Expression Analysis by Quantitative Reverse Transcription Real-Time PCR

Total RNA (200 ng) was used for qRT-PCRs of the miR-29c, miR-34a, miR-122, miR-155, miR-192, miR-200b, miR-203 and miR-221, utilizing TaqMan miRNA assays (Applied Biosystems, Foster City, Calif., USA), according to the manufacturer's instructions. snoRNA202 was used as an endogenous control. The relative amount of each miRNA was measured using the $2^{-\Delta\Delta Ct}$ method [12]. All qRT-PCR reactions were conducted in triplicate and repeated twice.

Gene Expression Analysis by qRT-PCR

Total RNA (10 μg) was reverse transcribed using random primers and a high-capacity cDNA archive kit (Applied Biosystems), according to the manufacturer's protocol. The expression of the α-smooth muscle actin *(α-Sma)* gene was measured by qRT-PCR, using Taqman® gene expression assay (Mm00725412_s1; Applied Biosystems).

Western Blot Analysis of Protein Expression

The levels of cyclin G1 (Ccng1), cyclogenase 2 (Cox2), E2F transcription factor 3 (E2f3), and CCAAT enhancer binding protein beta (C/ebp-β) proteins were determined by Western immunoblot analysis [13].

Statistical Analysis

Results are presented as mean ± SD. Statistical analyses were conducted by 1-way ANOVA, using treatment and weeks as fixed factors. Pair-wise comparisons were conducted by the Student-Newman-Keuls test. p values <0.05 were considered significant.

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Results and Discussion

Dysregulation of miRNAs in the Livers of C57BL/6J Mice Fed a Methyl-Deficient Diet

miRNA microarrays were used to analyze the miRNA expression profiles in the livers of control C57BL/6J mice and C57BL/6J mice fed a methyl-deficient diet that causes a liver pathological state similar to human nonalcoholic fatty liver disease [14]. We identified 74 miRNAs (40 up-regulated and 34 down-regulated) that were differentially expressed ($p < 0.05$), including miR-15a, miR-29c, miR-30a, miR-34a, miR-101a, miR-107, miR-122, miR-155, miR-200b, miR-200c, miR-221, miR-222 and miR-224 in the livers of the C57BL/6J methyl-deficient mice (fig. 1a). The results obtained by miRNA microarray analysis were confirmed by qRT-PCR (fig. 2a).

Functions of Dysregulated miRNAs

Dysregulated miRNAs are known to affect cell proliferation, apoptosis, lipid metabolism, oxidative stress, DNA methylation and inflammation. These processes are substantially compromised in pathological states associated with hepatocarcinogenesis. Specifically, it is well-established that altered lipid metabolism, oxidative stress, apoptosis and epigenetic alterations may directly trigger hepatic steatosis, a condition that has been shown to progress to hepatocellular carcinoma [15–17].

Among the down-regulated miRNAs, miR-15a, miR-30a, miR-101a and miR-122 are of particular interest. Previously, we and other investigators have demonstrated a substantial down-regulation of liver-specific miR-122 during liver carcinogenesis and in primary hepatocellular carcinomas [18–21]. Recently, a significant decrease in miR-122 expression has been observed in individuals with non-alcoholic steatohepatitis [22]. The down-regulation of miR-122 in the livers of C57BL/6J mice fed a methyl-deficient diet was accompanied by increased level of Ccng1 protein (fig. 1b). The altered expression of CCNG1 [19] and other confirmed targets of miR-122, such as fatty acid synthase [22, 23], sterol regulatory element-binding protein-1c [22, 23], cationic amino acid transporter (CAT1; SLC7A1) [24], and BCL-W, an anti-apoptotic member of BCL2 family member [25], has frequently been observed during hepatocarcinogenesis and has been attributed to the pathogenesis of liver cancer.

Feeding C57BL/6J mice a methyl-deficient diet for 12 weeks resulted in decreased expression of miR-101a and miR-101b (fig. 1a). One of the confirmed targets for miR-101a is Cox-2 [26], which is substantially up-regulated in the livers of mice exposed to the methyl-deficient diet (fig. 1b). The increased expression of COX-2 has been detected during human and rodent liver tumor development [27, 28] and is currently considered as an attractive target for chemoprevention during early stages of hepatocarcinogenesis. Additionally, recent evidence has demonstrated that miR-101 targets FBJ murine osteosarcoma viral oncogene homolog (FOS) oncogene [29], a key component of the liver oncogenic network [30].

Another down-regulated miRNA in the livers of mice fed the methyl-deficient diet is miR-15a, one of the first miRNA's discovered to be dysregulated in cancer [31].

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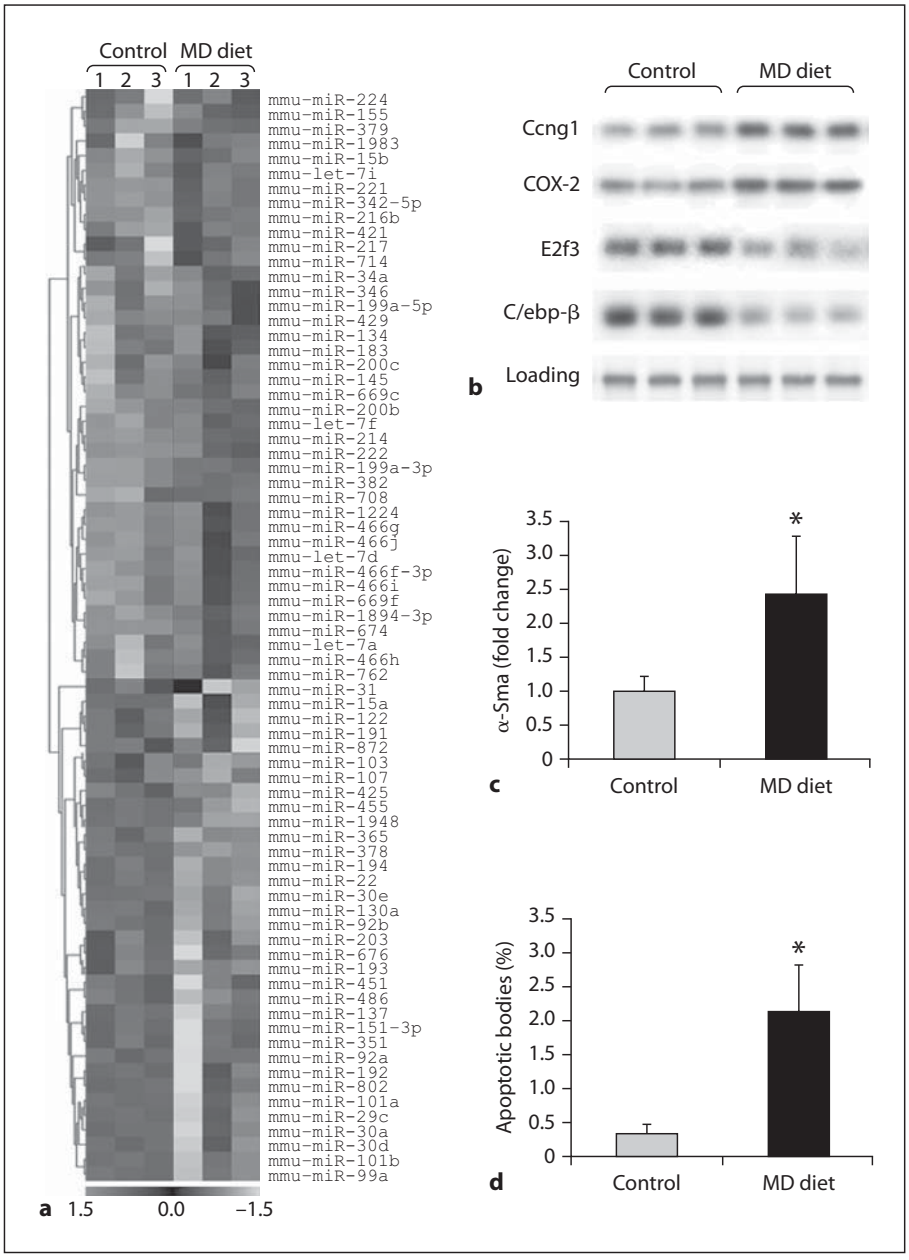

Fig. 1. Dysregulation of miRNA expression in the livers of C57BL/6J mice fed a methyl-deficient diet for 12 weeks. **a** Hierarchical clustering of the differentially expressed miRNA genes (as determined by ANOVA) in the livers of control and methyl-deficient (MD) mice. Rows show miRNA, while columns show independent biological replicates. For each miRNA red indicates high expression levels and green indicates low expression levels. Each miRNA listed is significantly differentially expressed ($p < 0.05$; $n = 3$). **b** Western blot analysis of Ccng1 (miR-122), COX-2 (miR-101a), E2f3 (miR-34a and miR-200b) and Cebp/β (miR-155) proteins in the livers of control and methyl-deficient mice. **c** qRT-PCR analysis of *α-Sma* gene in the livers of control and methyl-deficient mice (mean ± SD; $n = 5$). **d** Apoptotic cell death in the livers of control and methyl-deficient mice as detected by TUNEL assay (mean ± SD; $n = 5$).

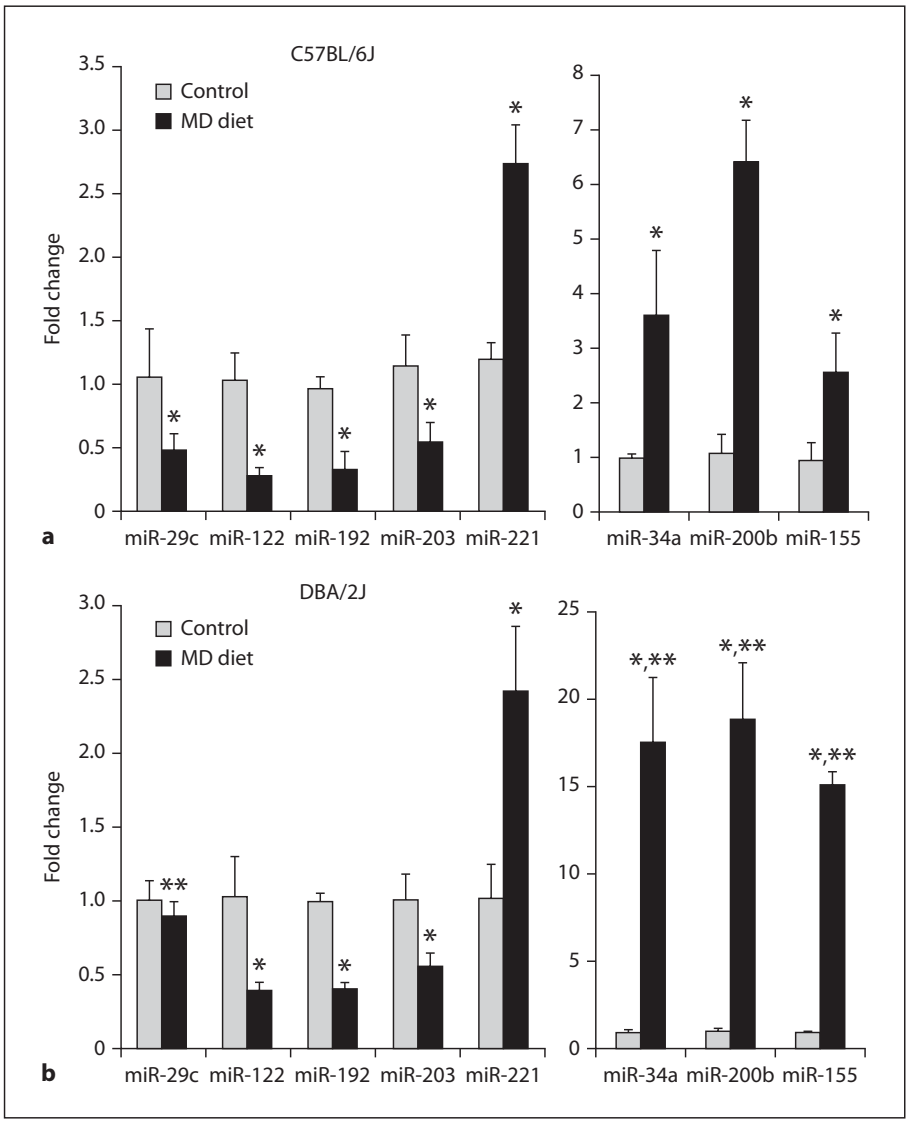

Fig. 2. qRT-PCR analysis of differentially expressed miRNAs in the livers of control C57BL/6J (**a**) and DBA/2J mice (**b**) and mice fed a methyl-deficient diet (MD) for 12 weeks. * Significantly different from control mice. ** Significantly different from C57BL/6J methyl-deficient mice (mean ± SD; n = 5).

miR-15a targets multiple oncogenic pathways, including BCL2, cyclin D1 (CCND1) and WNT3A signaling [31], a pathway that triggers the activation of hepatic stellate cells and progression of hepatic fibrosis [32]. miR-107 [20] and let-7a and let-7d [33], which are down-regulated (miR-107) and up-regulated (let-7a and let-7d) in the livers of methyl-deficient mice (fig. 1a), have also been associated with the pathogenesis of hepatic steatosis, fibrosis and hepatocarcinogenesis. Indeed, figure 1c shows an increase in expression of the *α-Sma* gene, a marker of hepatic stellate cell activation and fibrosis development [34] in the livers of mice fed the methyl-deficient diet.

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miR-34a, miR-155, miR-200b and miR-221 were the most up-regulated miRNAs among the differentially expressed miRNAs in the livers of methyl-deficient C57BL/6J mice (figs. 1a and 2). The transcription factor E2f3, a critical regulator of the p53 network, is one of the targets for these miRNAs as reported in Targetscan 5.1 (www.targetscan.org) and in other reports [35, 36]. Furthermore, there is a solid connection between miR-34 and the p53 apoptotic pathway [37–39], which plays a pivotal role in the pathogenesis of liver injury regardless of its etiology, and especially in non-alcoholic hepatosteatitis [40, 41]. Figure 1d shows the increased apoptosis in the livers of C57BL/6J mice fed a methyl-deficient diet. Additionally, recent evidence has demonstrated the importance of miR-34a, not only in apoptosis, but also in non-apoptotic cell death in vivo [42].

The over-expression of miR-155 and miR-221 has been frequently detected during tumor development [43, 44]. The up-regulation of these miRNAs has been associated with activation of the extracellular signal-regulated (ERK) and phosphatidylinositol 3-kinase (PI3)-AKT pathways, 2 pathways frequently disturbed during liver tumorigenesis. Furthermore, the results of a recent study have demonstrated that miR-221 targets and down-regulates pro-apoptotic BCL2-modifying factor during human hepatocarcinogenesis [45]. It is well-established that one of the hallmarks of the carcinogenic process is a dysregulation of cell proliferation and apoptosis [46]. In this context, the altered expression of miR-34a, miR-155, miR-200b and miR-221 in the livers of methyl-deficient mice illustrates the critical role of miRNA in the disruption of the delicate balance between cell division and apoptosis during carcinogenesis.

In a previous study [17], we demonstrated that feeding DBA/2J mice a lipogenic methyl-deficient diet resulted in more prominent pathomorphological and molecular changes in the livers, including DNA hypomethylation, a greater severity of steatosis and necrosis, and oval cell proliferation, as compared to C57BL/6J mice. Interestingly, we detected strain-specific significant differences in the expression of miR-29c, miR-34a, miR-155 and miR-200b in the livers of C57BL/6J (fig. 2a) and DBA/2J methyl-deficient mice (fig. 2b). Specifically, the expression of miR-34a, miR-155 and miR-200b in the livers of DBA/2J mice fed the methyl-deficient diet was, respectively, 4.9, 5.9 and 3.0 times greater than in methyl-deficient C57BL/6J mice. Likewise, the livers of C57BL/6J mice were characterized by a more pronounced down-regulation of miR-29c. The aberrant expression of these miRNAs is associated with an altered DNA methylation status (miR-29c), increased cell death (miR-34a and miR-200b), and liver steatosis and fibrosis (miR-155). miR-155, which was the most differentially expressed miRNA in the livers of DBA/2J and C57BL/6J mice fed the methyl-deficient diet, activates the AKT signaling pathway [47], triggering oval cell proliferation [48], a fundamental event in hepatocarcinogenesis.

In conclusion, these findings demonstrate that alterations in expression of miRNAs are a prominent event during early stages of liver carcinogenesis induced by methyl deficiency and strongly suggest that differences in the susceptibility to liver carcinogenesis may be determined by the variations in miRNA expression response. More

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importantly, our data provide a mechanistic link between alterations in microRNA expression and the pathogenesis of liver cancer.

Disclaimer

The views presented in this chapter do not necessarily represent those of the US Food and Drug Administration.

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