

Induction of microRNAome deregulation in rat liver by long-term tamoxifen exposure[☆]

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Abstract

Micro RNAs (miRNAs) are small non-coding RNA molecules that function as negative regulators of gene expression. They play a crucial role in the regulation of genes involved in the control of development, cell proliferation, apoptosis, and stress response. Although miRNA levels are substantially altered in tumors, their role in carcinogenesis, specifically at the early pre-cancerous stages, has not been established. Here we report that exposure of Fisher 344 rats to tamoxifen, a potent hepatocarcinogen in rats, for 24 weeks leads to substantial changes in the expression of miRNA genes in the liver. We noted a significant up-regulation of known oncogenic miRNAs, such as the 17-92 cluster, miR-106a, and miR-34. Furthermore, we confirmed the corresponding changes in the expression of proteins targeted by these miRNAs, which include important cell cycle regulators, chromatin modifiers, and expression regulators implicated in carcinogenesis. All these miRNA changes correspond to previously reported alterations in full-fledged tumors, including hepatocellular carcinomas. Thus, our findings indicate that miRNA changes occur prior to tumor formation and are not merely a consequence of a transformed state.

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent human cancer and is showing an increased incidence worldwide [1,2]. Understanding molecular mechanisms involved in hepatocyte transformation and identification of biological markers for early detection

of HCC are crucial for the determination of treatment strategies, as well as for chemoprevention [1,3].

Epigenetic mechanisms, which involve DNA methylation, histone modifications, and RNA-mediated silencing, resulting in the heritable alteration of expression of genes without a change in DNA sequence, have become increasingly recognized as important factors contributing to cancer development [4]. While changes in the levels of DNA methylation, histone modifications and gene expression during hepatocarcinogenesis induced by different carcinogenic agents have been intensively studied [1,3,5–8], the alterations in microRNAs (miRNAs) have remained unexplored. miRNAs are evolutionally conserved small single-stranded

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non-protein-coding RNA molecules presently recognized as major negative gene regulators [9–11]. In mammals, miRNAs negatively regulate their targets by either binding to imperfect complementary sites within the 3'-untranslated regions of their mRNA-targets [11], or by targeting specific cleavage of homologous mRNAs [12]. In the first case, miRNAs reduce protein levels of target genes by post-transcriptionally repressing target-gene expression without affecting mRNA levels of these genes, whereas in the second case, miRNAs induce the degradation of target mRNAs. In addition, recent findings suggest that miRNAs may act as regulators of various epigenetic processes [13–15].

miRNAs play key roles in diverse cellular processes, including development, cell proliferation, apoptosis, and the response to stress [9,10], and also function as tumor suppressors or oncogenes [16,17]. Emerging evidence suggests that miRNAs are involved in human disease, especially in oncogenesis [17–19]. Altered levels of miRNAs have been reported in a variety of human cancers [18,19] including HCC [20], and altered expression of specific miRNAs may be involved in tumorigenesis [21]. However, it has not yet been established whether miRNA changes found in tumors play a causative role in carcinogenesis or are merely a consequence of the transformed state. Additionally, the potential for the induction of miRNAs by carcinogens has not been studied.

Tamoxifen is a widely used drug for chemotherapy and, more recently, for the chemoprevention of breast cancer worldwide [22]. However, a number of studies have demonstrated that tamoxifen is hepatocarcinogenic in rats [23] with both tumor initiating [24] and promoting [25] properties. The mechanism of cancer initiation of tamoxifen in rats is due to the genotoxic reactivity of tamoxifen metabolism products resulting in formation of tamoxifen–DNA adducts [26]. The promoting activity of tamoxifen is associated, in part, with its ability to induce cell proliferation in liver [27], which certainly contributes to carcinogenic potential of tamoxifen.

In a previous study of tamoxifen-induced hepatocarcinogenesis in rats, we demonstrated substantial genotoxic and global epigenetic changes at early stages of carcinogenesis, characterized by decreased cytosine DNA methylation, decreased histone H4 lysine 20 trimethylation, and altered activity and expression of maintenance and de novo DNA methyltransferases [8]. Considering the intimate link between all components of the epigenetic regulatory system and the results of recent studies showing interdependent changes between miRNA levels and chromatin organization [13–15], the

current study was undertaken to determine whether or not tamoxifen administration could perturb miRNA expression in liver, thus predisposing the liver to carcinogenesis.

We demonstrate that long-term exposure of female Fisher 344 (F344) rats to a tamoxifen-containing diet leads to alterations in the miRNA expression profile in liver tissue prior to tumor formation. Importantly, we identified 33 miRNAs that are differentially expressed in the livers of tamoxifen-treated rats compared to age-matched control rats. Furthermore, we confirm the corresponding changes in the expression of some confirmed miRNA targets, which include important oncogenes, chromatin modifiers, and expression regulators implicated in carcinogenesis.

2. Materials and methods

2.1. Animals, treatment, and tissue preparation

Weanling female F344 rats were obtained from the National Center for Toxicological Research breeding facility, housed in a temperature-controlled (24 °C) room with a 12 h light–dark cycle, and given *ad libitum* access to water and NIH-31 laboratory diet. At 6 weeks of age, the rats (mean body weight 150 g) were allocated randomly to receive either NIH-31 diet containing 420 ppm tamoxifen (Dyets, Inc., Bethlehem, PA) or control NIH-31 diet. Diets were stored at 4 °C and given *ad libitum* with biweekly replacement. Five rats per diet group and five rats per control group were sacrificed at 6, 12, 18 and 24 weeks after diet initiation [8]. The livers were excised, frozen immediately in liquid nitrogen, and stored at –80 °C for subsequent analysis. All animal experimental procedures were carried out in accordance with animal study protocols approved by the National Center for Toxicological Research Animal Care and Use Committee. Taking into consideration that the highest level of the tamoxifen–DNA adducts and the most pronounced epigenetic changes were observed after 24 weeks of tamoxifen exposure [8], in the present study we analyzed the expression of miRNAs microarray analysis in liver of tamoxifen-fed rats and age-matched control rats at this specific time point.

2.2. miRNA microarray expression analysis

Total RNA was extracted from rat liver tissues using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions. miRNA microarray analysis was performed by LC Sciences (Houston, TX). Ten micrograms of total RNA were size-fractionated (<200 nucleotides) by using a mirVana kit (Ambion, Austin, TX). Poly-A tails were added to the RNA sequences at the 3' ends using a poly(A) polymerase, and nucleotide tags were then ligated to the poly-A tails. The tagged RNAs were hybridized to the dual-channel microarray μ ParaFlo microfluidics chips (LC Sciences) con-

taining 439 miRNA probes to rat and mouse miRNAs and then labeled with tag-specific dendrimer Cy3 and Cy5 fluorescent dyes. Dye switching was performed to eliminate the dye bias. The melting temperature of the detection probes was balanced by incorporation of varying numbers of modified nucleotides with increased binding affinities. Hybridization images were collected using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA) and digitized using Array-Pro image analysis software (Media Cybernetics, Silver Spring, MD). The maximum signal level of background probes was 180. A miRNA detection signal threshold was defined as twice the maximum background signal. Normalization was performed with a cyclic LOWESS (locally weighted regression) method to remove system-related variations [28]. Data adjustments included data-filtering, log 2 transformation, and gene centering and normalization. t-Test analysis was conducted between control and tamoxifen-treated sample groups. miRNAs with p -values < 0.05 were selected for cluster analysis. The clustering analysis was performed with a hierarchical method and with average linkage and Euclidean distance metrics [29].

2.3. Quantitative real-time PCR (qRT-PCR) expression analysis

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-16, miR-17-5p, miR-20, miR-22, miR-28, miR-34, miR-92, miR-106a, and miR-152, human 5S rRNA served as the positive control. qRT-PCR was performed on a SmartCycler (Cepheid, Sunnyvale, CA). End-point reaction products were also analyzed on a 3.5% high-resolution agarose gel stained with ethidium bromide. The results presented as fold change of each miRNA normalized to that of 5S rRNA in liver of tamoxifen-treated rats relative to control rats.

2.4. Western immunoblotting

Liver tissue samples were sonicated in 400–800 μ l of ice-chilled 1% sodium dodecyl sulphate (SDS) and boiled for 10 min. Small aliquots (10 μ l) of homogenate were reserved for protein determination using protein assay reagents from BioRad (Hercules, CA). Equal amounts of proteins (20 μ g) 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 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), E2F1 (1:500, Labvision Neomarkers, Fremont, CA), RB1 (1:750, Labvision Neomarkers), and NOTCH1 (1:250, Abgent, San Diego, CA). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology)

and an ECL Plus immunoblotting detection system (GE Healthcare Biosciences). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT). Unaltered PVDF membranes were stained with Coomassie Blue (BioRad) and the intensity of the M_r 50,000 protein band was assessed as a loading control. Signals were quantified using NIH ImageJ 1.63 Software and normalized to β -actin.

2.5. Statistical analysis

Results are presented as mean \pm S.D. Statistical analyses were conducted by two-way ANOVA, using treatment and weeks as fixed factors, with pair-wise comparisons being conducted by the Student–Newman–Keuls test. p -Values < 0.05 were considered significant.

3. Results

3.1. Effect of tamoxifen on miRNA expression in liver

miRNA microarrays were used to analyze the miRNA expression profiles in liver tissue of rats exposed to a tamoxifen-containing diet and in age-matched control rats. We identified 33 miRNA genes conserved amongst humans, mice, and rats (20 up-regulated and 13 down-regulated) that were differentially ($p < 0.05$) expressed in the livers of tamoxifen-treated rats (Fig. 1 and Table 1). Furthermore, 14 of these miRNA genes were differentially expressed at a level of $p < 0.01$ (Table 1).

The results obtained by miRNA microarray analysis were independently confirmed by qRT-PCR. We analyzed the expression of differentially expressed miR-16, miR-17-5p, miR-20, miR-22, miR-28, miR-34, miR-92, miR-106a, and miR-152 miRNA genes in liver of tamoxifen-treated and age-matched control rats. All qRT-PCRs confirmed data obtained by microarray analysis.

In order to determine whether or not alterations in miRNA expression are related to the genotoxic effect of tamoxifen, we measured expression of miRNAs in liver of rats maintained on tamoxifen-containing diet for 12 and 24 weeks. Feeding tamoxifen-containing diet resulted in sustained alteration of miRNAs expression (Table 2). Previously, we have shown that at these time points liver tissue displayed intensive cell proliferation [30]. Expression of miRNAs was also measured in the kidneys and mammary gland after 12 and 24 weeks of experiment. The expression did not differ between the tamoxifen-exposed and age-matched control rats (data not shown).

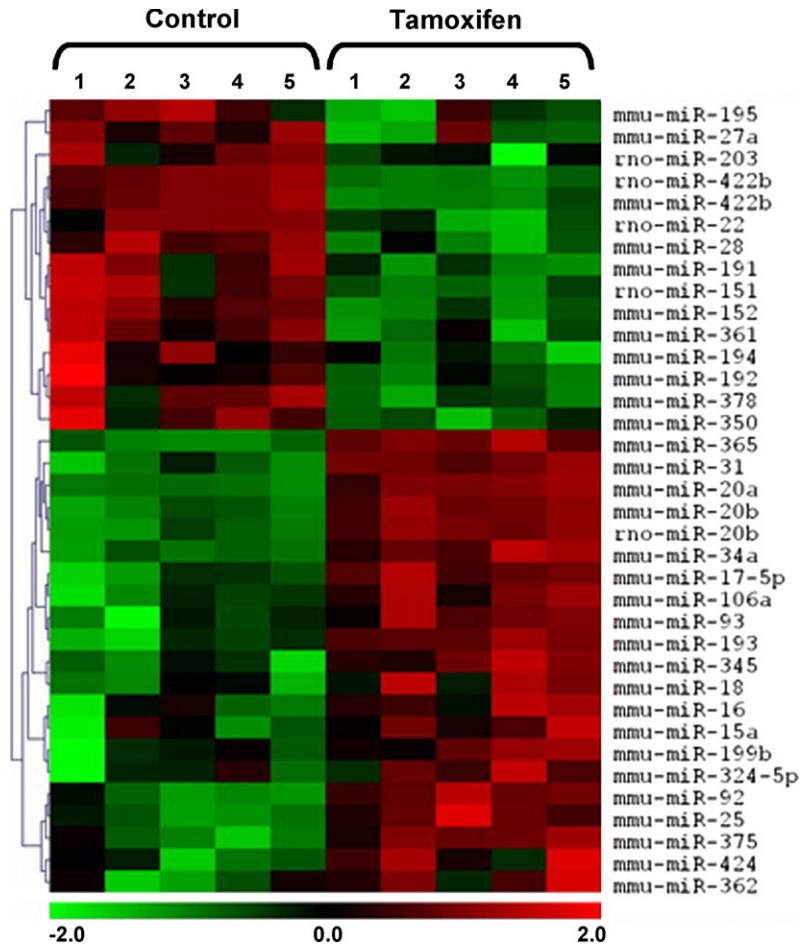


Fig. 1. Hierarchical clustering of differentially expressed miRNA genes in control and tamoxifen-treated liver samples after 24 weeks of tamoxifen-exposure. Hierarchical clusters of significantly altered miRNAs (as determined by ANOVA) across the tamoxifen-treated and control rats. Red denotes high expression levels, whereas green depicts low expression levels. Each miRNA listed is significantly differentially expressed ($p < 0.05$) between the tamoxifen-treated and control rats.

3.2. Expression of miRNA-targeted genes in liver of tamoxifen-treated and control rats

In view of the fact that miRNAs negatively regulate their gene targets and biological significance of miRNA deregulation depends on their protein-coding gene targets, we analyzed the protein level of confirmed targets of the differentially regulated miRNAs induced by tamoxifen available at <http://microrna.sanger.ac.uk>. Fig. 2 shows the level of proteins targeted by miR-16 (BCL2), miR-17-5p (E2F1), miR-20 (E2F1), miR-106a (RB1), and miR-34 (NOTCH1). The level of BCL2 and E2F1 proteins in the liver of tamoxifen-treated rats were decreased by 32% and 26%, respectively, compared to control rats. The most notable change was a decreased level of RB1 and NOTCH1 proteins by 56% and 45%, respectively, in the livers of rats fed

tamoxifen-containing diet, which was associated with an up-regulation of miR-106a and miR-34 (Table 2 and Fig. 2).

4. Discussion

In this report we describe that tamoxifen-induced hepatocarcinogenesis in rat is characterized by the substantial changes in expression of miRNA genes. The results of the study showed that that exposure of female F344 rats to tamoxifen resulted in deregulation of miRNA genes and their targets involved in cell cycle, DNA replication, chromatin maintenance, and apoptosis.

The RB family of tumor suppressors and the E2F family of transcription factors are involved in the maintenance of many cellular processes including regulation of the cell cycle, DNA replication,

Table 1
Differentially expressed miRNAs in liver of rats exposed to tamoxifen for 24 weeks

miRNA ^a	Species ^b	Fold change ^c
Up-regulated		
mmu-miR-34a	h, m, r	4.67 ^{***}
rno-miR-20b	r	2.18 ^{***}
mmu-miR-193	m, r	1.86 ^{**}
mmu-miR-106a	h, m, r	1.85 ^{**}
mmu-miR-16	h, m, r	1.82 [*]
mmu-miR-31	h, m, r	1.79 ^{***}
mmu-miR-345	h, m, r	1.71 ^{**}
mmu-miR-365	h, m, r	1.68 ^{***}
mmu-miR-17-5p	h, m, r	1.61 ^{***}
mmu-miR-20a	m, r	1.59 ^{***}
mmu-miR-375	h, m, r	1.48 ^{**}
mmu-miR-18	h, m, r	1.46 [*]
mmu-miR-424	h, m, r	1.45 [*]
mmu-miR-199b	h, m, r	1.42 [*]
mmu-miR-92	h, m, r	1.41 ^{***}
mmu-miR-25	h, m, r	1.40 ^{**}
mmu-miR-362	m, r	1.38 [*]
mmu-miR-93	h, m, r	1.35 ^{**}
mmu-miR-324-5p	m, r	1.34 [*]
mmu-miR-15a	h, m, r	1.23 [*]
Down-regulated		
mmu-miR-28	h, m, r	0.80 [*]
mmu-miR-191	m, r	0.79 [*]
mmu-miR-350	r	0.77 [*]
mmu-miR-361	h, m, r	0.73 [*]
rno-miR-22*	h, m, r	0.73 [*]
mmu-miR-27a	h, m, r	0.67 [*]
rno-miR-422b	r, m	0.62 ^{**}
mmu-miR-378	h, m, r	0.44 [*]
rno-miR-203	h, m, r	0.40 [*]
mmu-miR-195	m, r	0.36 [*]
rno-miR-152*	m, r, h	0.34 [*]
mmu-miR-192	h, m, r	0.25 [*]
mmu-miR-194	m, r	0.20 [*]

^a A total of 439 rat and mouse miRNAs were analyzed by miRNA microarray (LC Sciences).

^b Conserved in hsa (h), mmu (m), rno (r).

^c The data presented as fold change of the signal ratios of tamoxifen-treated rats to control rats. Dye switching was performed to eliminate the dye bias (see Section 2). These values represent the mean of five biological replicates.

* $p < 0.05$ compared to age-matched control rats.

** $p < 0.01$ compared to age-matched control rats.

*** $p < 0.001$ compared to age-matched control rats.

DNA-damage responses, DNA repair, and apoptosis [31,32]. Inactivation of RB1 and E2F1 proteins has been linked to tumorigenesis [31,32]. RB functions as a transcriptional repressor that regulates the expression of cell-cycle genes through its interaction with the E2F family of transcriptional factors [31] and/or by recruiting chromatin-modifying enzymes, such as histone deacetylases, histone methyltransferases, and DNA

Table 2
qRT-PCR analysis of miR-34a, -16, -17-5p, -20, -92, and -106a in liver of F344 rats exposed to tamoxifen for 12 and 24 weeks

miRNA	Fold change ^a		Confirmed targets
	12 weeks	24 weeks	
miR-34	4.2	7.0	NOTCH1
miR-16	1.8	1.8	BCL2
miR-17-5p	1.7	1.6	E2F1
miR-20	1.4	1.6	E2F1, TGFR β II
miR-92	1.3	1.3	E2F1
miR-106a	1.3	1.4	RB1

^a The data presented as average fold change of the signal ratios of each miRNA normalized to that of 5S rRNA of tamoxifen-treated rats to control rats. These values represent the mean of five biological replicates.

methyltransferase 1 to promoter regions [33,34]. The RB family of tumor suppressors also plays a major role in global chromatin structure [33]. Furthermore, a major role of the RB family proteins in the assembly of constitutive heterochromatin formation by maintaining histone H4 lysine 20 trimethylation at constitutive heterochromatin regions has recently been reported [33]. In our

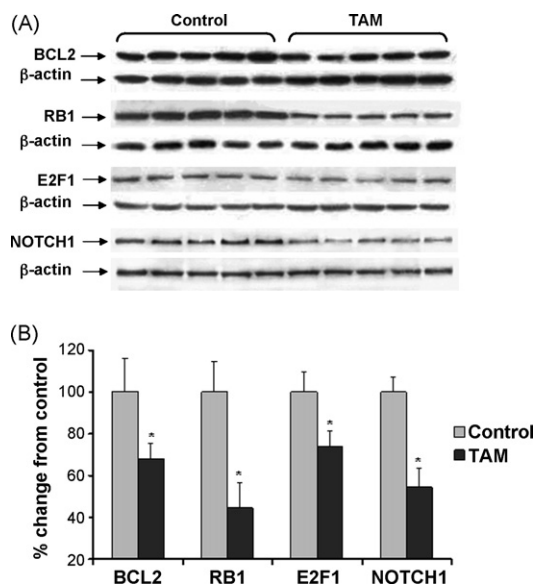


Fig. 2. Western blot analysis of the BCL2, RB1, E2F1, and NOTCH1 proteins in the livers of control and tamoxifen-exposed rats. (A) Liver tissue lysates were separated by SDS-PAGE and subjected to the immunoblotting using specific antibodies against the BCL2, RB1, E2F1, and NOTCH1 proteins. Equal sample loading was confirmed by immunostaining against β -actin. These results were reproduced in two independent experiments. (B) Quantitative evaluation of the BCL2, RB1, E2F1, and NOTCH1 proteins in the livers of control and tamoxifen-fed rats. Protein levels are presented as relative to age-matched control rats after normalization to β -actin ($n=5$, mean \pm S.D.). *Significantly different from control rats ($p < 0.05$).

previous study on the role of epigenetic mechanisms in the etiology of tamoxifen-induced hepatocarcinogenesis [8,30], we showed that exposure of rats to tamoxifen-containing diet resulted in a rapid and sustained decrease of histone H4 lysine 20 trimethylation. Considering this finding and the role of RB1 proteins in the heterochromatin maintenance, the increased expression of miR-106a and the associated decreased level of RB1 protein (Table 2 and Fig. 2) could result in a more “relaxed” heterochromatin organization, which could markedly increase genome instability and predispose liver cells to neoplastic transformation. This association between changes in level of RB1 protein and the status of histone H4 lysine 20 trimethylation provides additional evidence for the tight interaction between different components of the epigenetic system.

The NOTCH1 signaling pathway played an important role in controlling cell proliferation, differentiation, and apoptosis [35]. Recent evidence has indicated that NOTCH1 functions as a tumor-suppressor by inhibiting tumor growth through induction of cell cycle arrest and apoptosis [36,37]. Deregulation of NOTCH1 signaling has been implicated in the development of various cancers, including liver tumors [36,37]. In view of this, the elevated level of miR-34 expression and associated with it decreased level of NOCH1 protein in liver of tamoxifen-treated rats (Table 2 and Fig. 2) may mediate cell proliferation and provoke neoplastic cell transformation.

An additional interesting finding of this study is the up-regulation of the miR-16 and miR-17-92 cluster of miRNAs. miR-16 is a proven tumor-suppressor microRNA, up-regulation of which is associated with decreased level of BCL2 protein resulting in increased apoptosis [38]. In contrast, up-regulation of miR-17-92 cluster is associated with pro-proliferative and anti-apoptotic properties. This miRNA cluster, consisting of six miRNAs (miR-17-5p, miR-18, miR-19a, miR-19b, miR-20, and miR-92), is highly expressed in many human cancers [21]. Four miRNAs (miR-17-5p, miR-18, miR-20, and miR-92) from this cluster were significantly up-regulated in the livers of tamoxifen-treated rats (Fig. 1 and Table 2). Two miRNAs in this cluster, miR-17-5p and miR-20, directly regulate expression of the pro-proliferative transcription factor E2F1 [21]. Increased level of these miRNAs may alter E2F1 activity leading to promotion of cell proliferation and a decrease in cell death [21]. Furthermore, experimentally proven target for miR-20 is transforming growth factor β receptor II [39], inactivation of which may result in malignant cell transformation [40]. Considering the fact that miR-17-92 cluster acts primarily by suppress-

ing apoptosis [21,41], increased expression of miRNAs from this cluster may promote oncogenesis by enhancing cell proliferation and inhibiting apoptosis by escaping from the miR-15 and miR-16 anti-proliferative control resulting in neoplastic cell transformation. Indeed, our recent finding showing the elevated cell proliferation rate in the livers of tamoxifen-treated rats [30] supports this suggestion. In addition, a direct role of c-Myc in activation of miR-17-92 cluster has been shown recently [42]. In our previous study, we have detected a high level of c-Myc expression in liver of tamoxifen-treated rats after 12 and 24 weeks of exposure [30]. In light of these considerations, the ability of c-Myc to promote oncogenesis by driving unrestricted cell proliferation [43] may be related to its role in activation of miR-17-92 cluster.

A number of miRNAs, including miR-152 and miR-195, were also down-regulated in the livers of tamoxifen-treated rats. These miRNAs are frequently down-regulated in solid tumors [20,39].

Analysis of differentially expressed miRNA genes and their targets in liver of tamoxifen-exposed rats indicates that a majority of them are involved in regulation of cell cycle, DNA replication, and apoptosis. In our previous studies we showed the importance of the emergence of epigenetically reprogrammed cells as an indispensable preliminary event in liver carcinogenesis [8,30]. In view of this, tamoxifen-induced deregulation of expression of microRNA genes involved in cell cycle, DNA replication, chromatin maintenance, and apoptosis may be a crucial factor contributing to the emergence and maintenance of epigenetically reprogrammed cells resulting in neoplastic cell transformation. If this suggestion is correct, some of the differentially expressed miRNAs detected at early stages of carcinogenesis may persist in tumors. Indeed, it was recently reported that the miR-17-92 cluster was strongly up-regulated in HCC, while miR-195 was strongly down-regulated [20]. The elevated expression of miR-92, miR-20, and miR-18 was especially prominent in poorly differentiated HCC cells [20]. Additionally, a recent finding showing up-regulation of four microRNA genes, miR-106a, miR-17-5p, miR-20a, and miR-93, in liver tumors induced by a methyl-deficient diet [44] provides further support for this suggestion. The present study shows that the deregulation of miRNA genes expression occurs at early stages of liver carcinogenesis. More importantly, the finding of altered expression of the same miRNAs in liver tissue at early stages of carcinogenesis and in liver tumors, suggests that these changes may contribute to neoplastic cell transformation and be used as biomarkers for carcinogenic process.

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