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DMBA Induces Deregulation of miRNA Expression of *let-7*, *miR-21* and *miR-146a* in CBA/CA Mice

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Abstract. *Background: 7,12–Dimethylbenz(α)anthracene (DMBA) is a carcinogen capable of inducing various types of tumors. Materials and Methods: We investigated the effect of DMBA on micro-RNA expression in CBA/CA H2^k inbred mice after 24 hours and one week from exposure. Results: Expression levels of miR-21, miR-146a and let-7a were significantly higher in the vital organs of the mice 24 hours after DMBA exposure compared to those of the controls. On the other hand, a significant down-regulation of the miRNAs was observed seven days after DMBA administration. Conclusion: Based on our data, DMBA has an impact on the expression of miR-21, let-7a and miR-146a genes. The altered micro-RNA expression can be regarded as an early effect of exposure to chemical carcinogens. To our knowledge, this is the first study of miRNA modulation caused by DMBA in non-malignant tissues.*

There is some evidence that 7,12–dimethylbenz(α)anthracene can lead to the development of different types of tumors, such as lung cancer, lymphoma, leukemia, spleen hemangiosarcoma, skin and breast cancer (1-5). In our previous studies, we demonstrated up-regulated expressions of *Ha-ras, c-myc* and *p53* genes 24 and 48 hours after DMBA administration (6-8). Altered expression of these oncogenes and suppressor genes can be considered as a reliable indicator of tumorgenesis (9).

Micro-RNAs function as mediators of cell responses to extracellular signals by targeting genes involved in cell differentiation, proliferation and apoptosis. Deregulation of these short noncoding molecules plays an essential role in the multistep process of carcinogenesis. Recently several reports have indicated the dysregulation of miRNA in tumor formation and progression and their possible role in cancer diagnosis and therapy (10-13).

Let-7a is a member of the let miRNA family and is required for timing of cell fate determination. Temporal up-regulation of *let-7* miRNA in stem cells is required for their terminal differentiation at the adult stage (14). It has been reported that low expression of *let-7a* was strongly associated with different neoplasms, especially of the lung (15). Overexpression of *let-7* inhibited cell growth of a lung cancer cell line repressing *ras* and *c-myc* expression at translational level (15, 16).

miR-21 was found to be up-regulated in several types of human tumor. Suppressing the expression of several proapoptotic genes and *p53* tumor suppressor gene, *miR-21* contributes to the genesis and progression of many types of cancer (17, 18). Increased expression of *miR-21* has been detected in tumors of the breast, lung, pancreas, prostate, stomach and brain (19, 20).

It is clear that *miR-146a* plays an important role in the regulation of inflammatory responses through a negative feedback pathway suppressing the NFκB activity and the LPS induced inflammatory response (21). Up-regulated *miR-146a* targets several inflammation-related and membrane-associated messenger RNAs, including those encoding complement factor-H and the interleukin-1 receptor associated kinase-1, resulting in significant decreases in their expression (22).

Here we investigated the effect of intraperitoneal DMBA administration on *miR-21, let-7a* and *miR-146a* expressions in vital organs of CBA/CA mice.

Materials and Methods

5-Week-old CBA/CA H2k haplotype mice of both sexes were used. Animals weighed 20 g. Mice were divided into four groups (6 males and 6 females in each group). Two of the groups (group 1 and group 3) received intraperitoneal DMBA at a single 20 mg/kg animal weight dose (0.4 mg DMBA dissolved in 0.1 ml corn oil) at the start of the experiment. Group 2 and group 4 were the respective control

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groups, where animals consumed the standard laboratory chew pellet and tap water *ad libitum*. At 24 hours after DMBA administration, mice in the groups 1 and 2 were euthanized and autopsied. Seven days after DMBA exposure, the mice of group 3 and 4 were euthanized and autopsied. Liver, spleen, lungs and kidneys of the animals were removed during autopsy. Mice received humane care and the experiment was carried out under the approval of the Institutional Revision Board.

Tissue samples from the dissected organs were homogenized and miRNA was isolated with RNAzol solution (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's instruction. Quality of the isolated RNA was assessed by absorption photometry at 260/280 nm. Optical density of the RNA was between 1.9 and 2.1.

High purity miRNA was used in reverse transcription followed by nucleic acid amplification with a one-step RNA amplification kit: Light Cyler RNA Master SYBR Green I kit (Roche, Berlin, Germany) containing SYBR green fluorescent labeling. The PCR reaction mix included: 8.2 μl H_2O, 1.3 μl $Mn(OAc)_2$ stock solution, 7.2 μl LightCycler RNA master SYBR Green I fluorescent labeled dye, 2 μl specific primer at 0.5 μM final concentration and 1 μl template miRNA in 20 μl final volume. PCR amplifications were carried out in LightCycler 2.0 carousel based PCR system (Roche). PCR settings were the following: Reverse transcription at 61˚C for 20 minutes, pre-incubation (1 cycle) for 30 s at 95˚C, amplification (45 cycles): denaturation at 95˚C for 5 s, annealing at 50˚C for 15 s, extension at 72˚C for 5 s, melting curves (1 cycle) with denaturation at 95˚C for 0.1 s, annealing at 65˚C for 5s melting curve detection at 95˚C at 0.1 ramp rate for 8 s.

Sequence-specific primers for *let-7a, miR-21* and *miR-146a* were selected using the primer finder database (www.applied-science.roche.com) and were synthesized by TIB Molbiol, ADR Logistics, (Roche Warehouse, Budapest, Hungary): let-7a forward: 5'-GCCGCTGAGGTAGTAGGTTGTA-3', reverse: 5'-GTGCAGGGTCC GAGGT-3'; miR-21 forward: 5'-GCCCGCTAGCTTATCAGA CTGA TG-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'; miR-146a forward: 5'-GCCGCCCTGTGAAATTCAGTT-3', reverse: 5'-GTGCAGGGT CCGAGG -3'. Gene expression was determined by absolute nucleic acid quantification method in the case of miRNAs, with 4.0 Light Cycler software (Roche Diagnostics GmbH, Mannheim, Germany).

Student's *t*-test was performed between control and treated groups and *p*-values were calculated for each miRNA for each organ. *P*-Values less than 0.05 were considered statistically significant. Values were expressed as the mean±SD. Calculations were performed using Statistical Program for Social Science 19.0 (SPSS) software (IBM, Armonk, New York, USA).

Results

Expression levels of *miR-21, miR-146a* and *let-7a* isolated from DMBA-treated mice at the 24-hour time point and on the 7th day after DMBA treatment were compared with the values of the controls. The results after statistical analysis are shown in Table I.

There was a considerable up-regulation of all investigated miRNAs 24 hours after DMBA administration. *Let-7a* was found to be the most strikingly up-regulated miRNA, showing significantly higher levels of expression in all investigated tissues, particularly in the lung and kidney samples compared to the controls (Figure 1A and 1B). We also observed that *miR-21* was increased with a 2.64-fold change in the lung and 1.80-fold change in the spleen tissues (Figure 1A and 2B).

Table I. *Results of statistical analysis. Presented fold change values are the gene expression ratios of DMBA treated mice over the untreated controls according to tissue samples, duration of the treatment and miRNAs.*

	miRNA	Change in expression	Fold change	*p*-Value
Liver				
24 h	*let-7a*	Up	1.88	0.05
	miR-146a	Up	1.23	0.35
	miR-21	Up	1.10	0.96
1 week	*miR-21*	Down	0.38	0.24
	miR-146a	Down	0.45	0.47
	let-7a	Down	0.64	0.75
Spleen				
24 h	*let-7a*	Up	2.34	0.04
	miR-21	Up	1.80	0.06
	miR-146a	Up	1.12	0.71
1 week	*miR-146a*	Down	0.31	0.07
	miR-21	Down	0.46	0.44
	let-7a	Down	0.47	0.46
Lung				
24 h	*let-7a*	Up	2.90	0.01
	miR-21	Up	2.64	0.03
	miR-146a	Up	2.55	0.17
1 week	*let-7a*	Down	0.80	0.63
	miR-21	Down	0.82	0.63
	miR146a	Down	0.83	0.75
Kidney				
24 h	*let-7a*	Up	2.72	0.03
	miR-21	Up	1.44	0.60
	miR-146a	Up	1.26	0.65
1 week	*miR-21*	Down	0.67	0.10
	miR-146	Down	0.70	0.25
	let-7a	Down	0.98	0.71

In contrast to the 24-hour group, significant down-regulation of miRNAs was detectable on the 7th day after DMBA administration. *Let-7a, miR-21* and *miR-146a* expression in the liver and spleen were found to be significantly down-regulated one week after the exposure compared to controls (Figure 2A and 2B). In particular, the *miR-146a* expression in the spleen was more than three times lower than in the control (Figure 2B). In the lung and kidney, there were no statistically significant differences in the expression of the investigated miRNAs between the treated and the control animals on the 7th day after DMBA exposure (Figure 1A and 1B).

Discussion

Micro-RNAs can serve as mediators of cellular stress. Recently, many data have been published regarding the role of micro-RNA in response to environmental exposures in

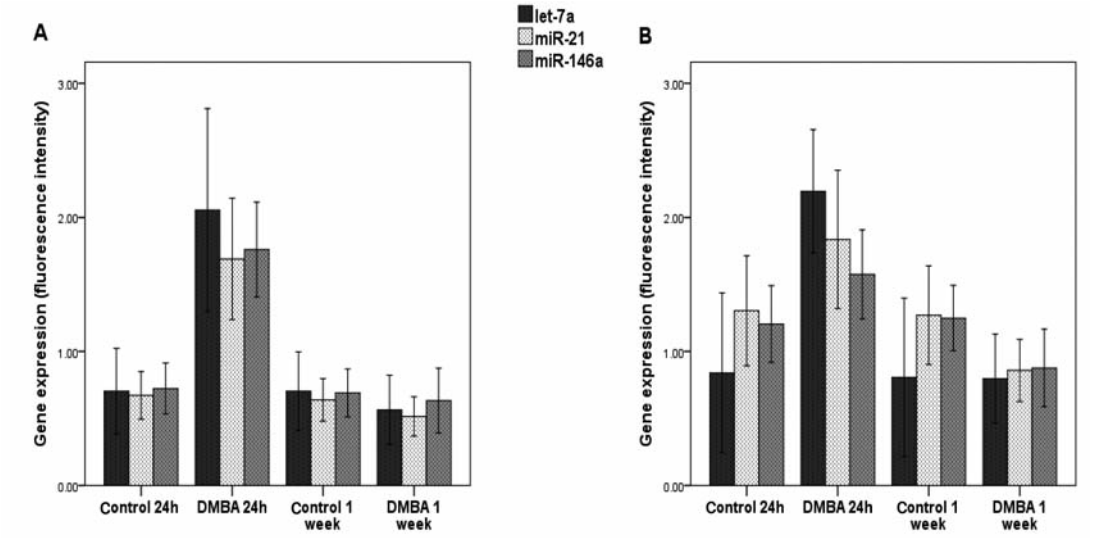

Figure 1. *let-7a, miR-21, miR-146a gene expression in lung (A) and kidney (B) of mice 24 h and one week after DMBA exposure compared to controls. Values are the mean of six mice±SD.*

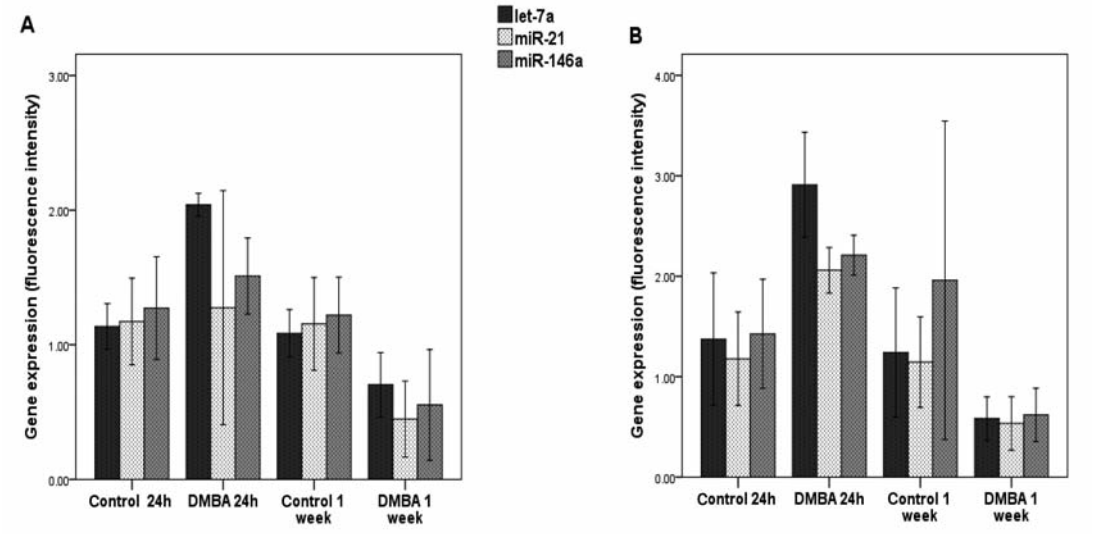

Figure 2. *let-7a, miR-21, miR-146a gene expression in liver (A) and spleen (B) of mice 24 h and one week of DMBA exposure compared to controls. Values are the mean of six mice±SD.*

non-malignant tissues (23). Some previous studies analyzed the effect of cigarette smoke on miRNA expression in lung tissues of rats. Izotti *et al*. demonstrated down-regulation of several miRNAs, including let-7, miR-30, miR-34, miR-140, miR-145, miR-146 and miR-192 family, genes controlling cell growth, differentiation and survival (24, 25). Another study reported that rats treated with the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone showed reduced expression of miR-34, miR-101, miR-126 and miR-199 in lung (26). According to Progribny *et al*., rats exposed to tamoxifen, a potential hepatocarcinogen, mainly showed up-regulation of miRNAs, functioning oncogenes in hepatocellular carcinogenesis, such as *miR-34a*, *miR-106* and *miR-20b* (27). When Zhang and Pan tested the effect of hexahydro-1,3,5–trinitro–1,3,5–triazine on the miRNA expression in mouse liver, there was significant increase in the levels of *miR-30e*, *miR-99a* and *miR-192*, and down-regulation of *let-7* family members (28). Based on these

studies, miRNA expression appears to be modified in response to various chemical agents. Such studies were carried out with long exposure periods, but there are no data available regarding the changes of miRNA levels induced by carcinogens in 24 hours.

We monitored the levels of *let-7a, miR-21* and *miR-146a* in mice at 24 hours and seven days after DMBA administration. Statistically increased levels were observed in the expression of the analyzed miRNAs 24 hours after DMBA administration in vital organs of the mice. While an opposite trend was observable in the case of the group that was investigated seven days after the same chemical exposure. In a recent study, Yu *et al*. induced oral cancer by 5% DMBA in Syrian hamsters and dysregulation of 17 miRNAs was observed in the tumor samples. They revealed the up-regulation of five miRNAs: *miR-21, miR-200b, miR-221, miR-338* and *miR-762* (29). Our study gave parallel results regarding onco-miR-21 at the 24-hour time point after DMBA administration.

In summary, the present study provides a model for the analysis of early miRNAs expression alterations after DMBA exposure. Our findings suggest that the overexepression of the investigated miRNAs might be involved in the postexposure effect of environmental carcinogens such as DMBA. A deeper understanding of miRNA regulation in chemically induced carcinogenesis opens the possibilities for the development of molecular biomarkers for screening and primary cancer prevention.

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