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Very Early Effect of DMBA and MNU on MicroRNA Expression

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Abstract. *Background: microRNA expression profile analysis provides evaluation of the early stages of carcinogenesis. This study focuses on early alteration of miRNA expression after treatment with different carcinogens. Materials and Methods: Mice were intraperitoneally injected with one dose of 7,12-dimethylbenz(α)anthracene (DMBA) and N-methyl-N-nitrosourea (MNU). The expression of miRNAs were analyzed 3 and 6 hours after the treatment, using quantitative real-time-polymerase chain reaction. Results: Underexpression of miR-34a and miR-155 were detected in the liver, spleen and kidneys at 3 and 6 hours after MNU treatment. In the lungs and kidneys, the expression of miR-21 was significantly elevated 6 hours after DMBA treatment, while in the liver, MNU induced higher expression levels of miR-21 at 3 and 6 hours compared to treatment with DMBA. Conclusion: The different response of miRNAs to carcinogens emphasizes their possible role as potential epidemiological biomarkers in early phases of environmental tumorigenesis.*

7,12-Dimethylbenz(α)anthracene (DMBA) and *N*-methyl-*N*-nitrosourea (MNU) are two important chemical carcinogens. There is evidence that a single dose of these agents is able to induce various types of cancer in animal models (1, 2). One of the major differences between the two agents is in their activation. DMBA is a polycyclic aromatic hydrocarbon which must be metabolized by P450 enzymes to become mutagenic, while MNU requires no metabolic activation (3). MNU serves as a tumor initiator by transferring methyl groups in DNA, whereas DMBA is involved in initiation, as well as in promotion, of tumorigenesis (4, 5). In previous studies, we reported that DMBA and MNU caused overexpression of *H-Ras*, *c-Myc* and *p53* genes 24-48 hours after the treatment (6, 7). Additionally, we compared the short-term effects (within 24 hours) of DMBA and MNU on the level of oncosupressor genes (8). We observed that overexpression of *c-Myc*, *H-Ras* and *p53,* induced by carcinogens, was strongly linked to the activation and metabolism of DMBA and MNU.

MicroRNAs (miRNAs) represent an newly emerging field of cancer research. By regulating gene expression, miRNAs play an important role in the control of cellular processes. To date, more than 17,000 miRNAs have been identified, out of which approximately 1,400 miRNAs can be found in humans (9). There is evidence that miRNA expression can respond very sensitively to carcinogen exposure, which provides a good model for investigating the acute effect of carcinogenic agents (10). Based on toxicological research focusing on miRNA expression, Chen demonstrated that the miRNA expression pattern can be specific to different types of exposure to carcinogens and their target tissues (11).

We previously investigated the acute effect (24 hours) of DMBA and MNU on miRNA expression in healthy tissues. We analyzed the alteration of miRNA expression induced by intraperitoneal injections of these carcinogens (12, 13). According to our results, there was a marked difference in miRNA expression detected at 24 hours with that at one week after the exposure to DMBA, but there was no significant difference between the two groups after MNU treatment.

The aim of this short-term experiment was to reveal the differences between DMBA-, and MNU-induced tumorigenesis at the level of miRNA expression. We selected miRNAs including *let-7a, miR-21, miR-34a, miR-146a* and *miR-155* to determine the modification in their expression after carcinogenic exposure *in vivo*.

Materials and Methods

Six groups of 6-week-old CBA/Ca $H2^k$ inbred mice constituted the experiment (Table I). Each group consisted of 6 male and 6 female animals, weighing 20-25 g. Two groups were treated intraperitoneally with a single dose of DMBA at the onset of the experiment. Another two groups of animals were given

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Table I. *Preparation of groups for this study.*

Group	Treatment	Autopsy
1	Consumption of laboratory chew pellet and tap water *ad libitum*	3 Hours after the onset of the examination
2	Consumption of laboratory chew pellet and tap water *ad libitum*	6 Hours after the onset of the examination
3	Intraperitoneal injection of DMBA in a single 20-mg/kg animal weight dose (0.4 mg DMBA dissolved in 0.1 ml corn oil)	3 Hours after the DMBA injection
4	Intraperitoneal injection of DMBA in a single 20-mg/kg animal weight dose (0.4 mg DMBA dissolved in 0.1 ml corn oil)	6 Hours after DMBA injection
5	Intraperitoneal injection of MNU in a single 30-mg/kg animal weight dose (0.6 mg MNU dissolved in 0.1 ml corn oil)	3 Hours after the MNU injection
6	Intraperitoneal injection of MNU in a single 30-mg/kg animal weight dose (0.6 mg MNU dissolved in 0.1 ml corn oil)	6 Hours after the MNU injection

intraperitoneal injection of MNU at the start of the investigation. Mice belonging to control groups received standard laboratory chew pellet and tap water *ad libitum*. At the 3- and 6-hour timepoints of the experiment, mice were euthanized and autopsied (Table I). The animal experimental procedures were carried out in accordance with the Institutional Revision Board.

During the autopsies, the liver, spleen, lungs and kidneys were removed and the tissue samples from the dissected organs were homogenized. Total RNA was extracted using TRIZOL reagent according to the manufacturer's protocol (Molecular Research Center Inc., Cincinnati, OH, USA). The quality of the isolated RNA was checked by absorption photometry at 260/280 nm. Total RNA was exposed to RNAase-free DNAase and 2 μl were reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Berlin, Germany).

The expression of the investigated miRNAs was determined by quantitative real-time polymerase chain reaction (PCR). PCR primers were designed using the primer finder database (www.applied-science.roche.com) and were synthesized by TIB Molbiol, ADR Logistics, (Roche Warehouse, Budapest, Hungary). The sequences of the primers are listed in Table II. The PCR was performed on a LightCycler 2.0 carousel-based PCR system (Roche, Berlin, Germany). The 20-μl PCR reaction mixture included: 7.2 μl LightCycler RNA master SYBR Green I fluorescent labeled dye, 1.3 μl $Mn(OAc)_2$ stock solution, 2 μl specific primer at 0.5 μM final concentration, 1 μl template miRNA and 8.2 μl H_2O. The reaction mixtures were incubated for 30 s at 95˚C, followed by 45 three-step amplification cycles (95˚C for 5 s, 50˚C for 15 s, 72˚C for 5 s). The statistical calculation of differences in expression was performed using the Statistical Program for Social Science 19.0 (SPSS) software (IBM, Armonk, NY, USA). Student's *t*-test was performed between control and treated groups, and *p*-values less than 0.05 were considered statistically significant.

Results

In the liver, MNU induced higher expression of *miR-21* at the 3- and 6-hour timepoints compared to the values detected in control and DMBA-treated groups (Table III). DMBA exposure significantly elevated the expression of *miR-21* in lungs and kidneys 6 hours after the treatment compared to MNU (Tables V and VI).

Except for the kidneys, the expression level of *miR-155* was elevated in DMBA-treated groups at the 3- and 6-hour timepoints in comparison with groups which received MNU treatment (Tables III-V). In the kidneys, there was no difference between DMBA and MNU-treated groups 3 hours after the exposure, while at the 6 hour timepoint, DMBA treatment resulted in a significantly higher expression level than did MNU (Table VI).

A comparison of *miR-34a* expression between DMBA- and MNU-treated groups revealed that it was significantly higher in liver and kidney tissues 3 and 6 hours after DMBA treatment and at 6 hours in spleen, while MNU induced a higher expression level of *miR-34a* in lungs compared to DMBA (Tables III-VI).

The expression of *let-7a* did not markedly differ between DMBA- and MNU-treated groups (Tables III, IV and VI). There was a relevant difference observed in the expression of *miR-146a* in the kidneys, where the expression levels detected in DMBA- and MNU-treated groups were found to decrease below those of the control groups (Table VI).

Discussion

With the recognition of the crucial role of miRNAs in carcinogenesis, there is increasing interest in the relationship between the miRNA expression profile and exposure to carcinogenic agents. Recently, several studies reported on the role of miRNAs in genotoxicogical research such as vinyl-carbamate, 2- acetylaminofluore, *N*-ethyl-*N*-nitrosurea (ENU), DMBA, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butatone, 1,3,5-trinitro-1,3,5-triazine, tamoxifen and cigarette smoke (14-20). In the recent study we paralelly investigated the impact of DMBA and MNU treatment on miRNA expression in mice.

According to our results, the exposure of mice to MNU resulted in lower expression of *miR-34a* compared to those of controls and DMBA-treated groups in all investigated organs except for the lungs. *miR-34a* is recognized as a tumor supressor whose activation is strongly linked to the p53

Table II. *PCR primer sequences*.

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
let-7a	GCCGCTGAGGTAGTAGGTTGTA	GTGCAGGGTCCGAGGT
miR-21	GCCCGCTAGCTTATCAGACTGATG	GTGCAGGGTCCGAGGT
miR-34a	TGGCAGTGTCTTAGCTGGTTG	GGCAGTATACTTGCTGATTGCTT
miR-146a	GCCGCCCTGTGAAATTCAGTT	GTGCAGGGTCCGAGG
miR-155	GTTAATGCTAATCGTGATAGGG	GTTAATGCTAATCGTGATAGGG

Table III. *Results of statistical analysis. Presented fold change values are the gene expression ratios of 7,12-dimethylbenz(α)anthracene (DMBA)- and N-methyl-N-nitrosourea (MNU)-treated mice, relative to the controls in liver.*

	MNU		DMBA	
	Fold change	*p*-Value	Fold change	*p*-Value
3 Hours				
let-7a	0.86	0.741030	0.96	0.922212
miR-21	6.75	0.004394	2.92	0.097368
miR-146a	3.64	0.087430	0.23	0.046985
miR-155	0.35	0.015657	1.68	0.133346
miR-34a	0.37	0.017707	1.09	0.681986
6 Hours				
let-7a	1.73	0.253374	1.89	0.162093
miR-21	9.54	0.001375	7.03	0.001375
miR-146a	3.82	0.064963	3.11	0.102677
miR-155	0.04	0.000714	2.18	0.000492
miR-34a	0.14	0.003084	1.81	0.024370

Table IV. *Results of statistical analysis. Presented fold change values are the gene expression ratios of 7,12-dimethylbenz(α)anthracene (DMBA)- and N-methyl-N-nitrosourea (MNU)-treated mice, relative to the controls in spleen.*

	MNU		DMBA	
	Fold change	*p*-Value	Fold change	*p*-Value
3 Hours				
let-7a	5.20	0.067843	3.60	0.076096
miR-21	3.06	0.001408	3.92	0.001408
miR-146a	46.00	0.012181	83.00	0.009116
miR-155	0.28	0.000799	1.05	0.798956
miR-34a	0.36	3.24417E-05	0.31	5.45962E-05
6 Hours				
let-7a	14.60	0.005089	9.00	0.005089
miR-21	3.72	0.083721	3.38	0.005598
miR-146a	18.00	0.007723	53.00	5.43336E-06
miR-155	0.07	0.000108	0.71	0.085430
miR-34a	0.24	2.05268E-05	0.98	0.955927

Table V. *Results of statistical analysis. Presented fold change values are the gene expression ratios of 7,12-dimethylbenz(α)anthracene (DMBA)- and N-methyl-N-nitrosourea (MNU)-treated mice, relative to the controls in lungs.*

	MNU		DMBA	
	Fold change	*p*-Value	Fold change	*p*-Value
3 Hours				
let-7a	1.53	0.118577	0.31	0.053351
miR-21	1.21	0.658663	1.66	0.365204
miR-146a	0.54	0.242364	0.95	0.929095
miR-155	0.31	0.027857	0.69	0.414987
miR-34a	1.22	0.636319	0.75	0.409987
6 Hours				
let-7a	0.42	0.094777	1.81	0.113964
miR-21	2.58	0.009116	8.21	0.000407
miR-146a	0.99	0.992283	0.58	0.245629
miR-155	0.09	0.007292	0.71	0.316884
miR-34a	0.97	0.950365	0.67	0.275195

Table VI. *Results of statistical analysis. Presented fold change values are the gene expression ratios of 7,12-dimethylbenz(α)anthracene (DMBA)- and N-methyl-N-nitrosourea (MNU)-treated mice, relative to the controls in kidneys.*

	MNU		DMBA	
	Fold change	*p*-Value	Fold change	*p*-Value
3 Hours				
let-7a	0.26	0.004908	0.17	0.002115
miR-21	1.36	0.466178	1.11	0.741001
miR-146a	0.01	0.008117	0.09	0.014008
miR-155	0.43	0.000159	0.44	0.002764
miR-34a	0.31	1.98602E-05	0.57	0.007420
6 Hours				
let-7a	0.44	0.043532	0.22	0.003423
miR-21	1.28	0.043532	3.09	0.000972
miR-146a	0.02	0.009240	0.03	0.008813
miR-155	0.03	7.40465E-08	0.49	0.001490
miR-34a	0.24	3.12669E-05	0.81	0.070701

pathway (21). *miR-34a* targeting *B-cell lymphoma-2* (*BCL2*), *cyclin D1*, *MYCN*, *cyclin-dependent kinase-4* (*CDK4*), *sirtuin-1* (*SIRT1*) genes can influence all steps of carcinogenesis (22, 23). Chen *et al*. investigated the acute effect of N-ethyl-N-nitrosurea on miR-34a expression in mice (24). ENU, similarly to MNU, acts as an alkylating agent. In contrast to our results, they showed that exposure of mice to ENU induced a markedly elevated level of *miR-34a* after 24 hours of treatment in spleen. When Le *et al*. exposed rats to ENU for 1, 3, 7, 15, 30 and 120 days, they detected an elevated level of *miR-34* family in mouse liver 7 and 15 days after the treatment (25).

Based on the differences in miRNA expression between controls and treated groups, the effect of MNU appeared more dominant on *miR-21*, *miR-34a* and *miR-155* expression compared to DMBA. These very early responses of miRNAs in the MNU-treated groups can be explained by the fact that MNU is a direct-acting carcinogen, while DMBA is an indirect carcinogenic agent. Metabolic activation of DMBA by cytochrome P450 enzymes, which are mainly synthesized in liver, may explain the higher expression levels of *miR-34a* and *miR-155* in liver of DMBA-treated mice (26).

In the investigated organs, exposure to DMBA induced significantly higher expression levels of *miR-155* than MNU. *miR-155* has been identified as an oncomiR contributing to the developement of hematopoietic malignancies (27). Increased expression of *miR-155* was shown in different types of human cancer by targeting tumor supressor genes including *tumor protein p53 inducible nuclear protein-1* (*TP53INP1*) and *RhoA* (28). Similarly to *miR-155*, *miR-21* functions as oncogenic miRNA. Negatively regulating the expression of *p53*, *phosphatase and tensin homolog* (*PTEN*), *programmed cell death-4* (*PDCD4*) and *tropomyosin-1* (*alpha*) (*TPM1*) genes *miR-21* can contribute to the process of carcinogenesis (29, 30). *miR-21* was expressed at a higher level in mouse liver after MNU treatment compared to DMBA, while DMBA induced the overexpression of *miR-21* 6 hours after the treatment in lung and kidney tissues compared to MNU.

There was no considerable difference between the effect of the two agents on *let-7a* expression except for the lungs. While up-regulation of *let-7a* was detected at the 6-hour timepoint in lungs after DMBA treatment, an opposite tendency was observed at the 3-hour timepoint. Repression of *c-Myc* and *RAS* genes by *let-7a* can contribute to pathogenesis of different types of human cancer such as tumors of lung, colon, stomach and breast (31, 32).

In conclusion, in the current study we provided evidence that DMBA and MNU differentially altered the expession of *miR-21, miR-34a* and *miR-155*, and the changes in their expression is strongly linked to the activation of carcinogens. The early alteration in the expression levels of the investigated miRNAs induced by carcinogenic agents (DMBA, MNU) support their role in initial processes of chemical carcinogenesis. Our results suggest that further analysis of miRNA expression shows promise for explanation of the early stage of chemically-induced tumorgenesis and primary tumor prevention based on miRNAs as early biomarkers.

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