GESDAV

Journal of Environmental and Occupational Science

available at www.scopemed.org

Original Research

Effect of N-methyl-N-nitrosurea on microRNA expression in CBA/CA mice

Krisztina Juhasz[1], Katalin Gombos[1], Katalin Gocze[1], Veronika Wolher[1], Monika Szirmai[1], Peter Revesz[2], Ingrid Magda[1], Andor Sebestyen[1], Istvan Ember[1]

[1]*Institute of Public Health, Faculty of Medicine, University of Pécs, Pécs, Hungary;*
[2]*Otorhinolaryngology and Head and Neck Surgery Clinic, Clinic Center, University of Pécs, Pécs, Hungary*

Received: June 17, 2012

Accepted: June 27, 2012

Published: July 22, 2012

DOI : 10.5455/jeos.20120627024402

Corresponding Author:
Krisztina Juhász, University of Pécs
Institute of Public Health
krisztina.juhasz01@gmail.com

Key words: N-methyl-N-nitrosourea, chemical carcinogen, chemically induced tumorgenesis, miRNA

Abstract

Aim: There is emerging evidence concerning the postexposure effect of chemical carcinogens on miRNA expression. In spite of this fact there haven't been so many investigation so far regarding the miRNA modification induced by N-methyl-N-nitrosourea, a well known pluripotent enviromental carcinogen. The aim of the study was to evalute the acute effect of MNU on miRNA expression. Methods: We investigated the expression level of miRNAs include miR-21 oncomiR and miR-146a is involved in NFkB antiapoptotic pathway and let-7a functioning as tumor supressor targeting ras and c-myc genes. The miRNAs expression levels were determined, in the year 2011, using quantitative PCR methods in CBA/CA $H2^k$ inbred mice at 24th hour and one week after MNU treatment and the data were evaluated in 2012. Results: We found significant upregulation of miR-21 gene in liver -, spleen – and kidney tissues. The most strikingly difference was observed in the the spleen, where all miRNA genes were upregulated in both treated groups related to the controls. Let-7a was also highly expressed in the most of the investigated organs, while miR-146a showed a reduction after 24 hours as well as one week later MNU injection. Conclusions: The early alteration in expression of let-7a, miR-21 and miR-146 genes in „in vivo" animal model could indicate their involvement in the acute effect of MNU, especially in the spleen. The characterisation of miRNA profile will lead to better understanding of mechanisms underlying the development of chemically induced tumorgenesis and may even serve as possible tool for indicate carcinogen exposures.

INTRODUCTION

N-methyl-N-nitrosourea (MNU) is a DNA alkylating agent which can cause GC to AT transition in DNA by formation of O6 methyl guanine adducts [1]. Acting as genotoxic carcinogen N-methyl-N-nitrosurea can induce several type of tumors such as alveolar adenocarcinomas, mammary tumors, malignant lymphomas of the thymus, liver and spleen [2-5]**.** Ando et al. demonstrated**,** that mice carrying human hybrid c-Ha-ras genes treated with single dose of MNU developed forestomach papillomas and skin papillomas [6] To evaluate the effect of MNU treatment on carcinogenesis, our research group have already investigated the expression of onco/supressor genes such as c-myc, Ha-ras and p53 genes 12 and 24 hours after the exposure in mice [7]. The results of this study supported, that MNU has an impact on the expression of c-myc, Ha –ras and p53 genes especially in 12 hours.

The microRNAs represent a group of small noncoding RNAs, whose mature form are 18-25 nucleotides in length. MiRNAs function as posttransciptional gene regulators, having a crurical importance in cell differentation, proliferation and apoptosis [8]. Over the past several years it has become clear that dysregulation of these small RNA molecules are involved in carcinogenesis. Number of studies have demonstrated altered expression of miRNAs in different type of human cancers [9-10]. Due to their complex role in gene regulation, miRNAs are implicated in cellular stress responses to extracellular signals such as chemical carcinogens [11]. The modification of miRNA expression induced by many

genotoxic agents like environmental cigarette smoke, N-ethyl-N-nitrosurea (ENU), 4- (methylnitrosamino)- 1- (3- pyridyl)- 1- butanone, hexahydro- 1,3,5- trinitro- 1, 3, 5- triazine (RDX), tamoxifen, 2- acetylaminofluorene, vinyl carbamate (VC), 7,12–dimethylbenz(α)anthracene (DMBA) is well documented [12-19]. Previously we provided data that exposure of mice to 7,12–dimethylbenz(α)anthracene results in significant overexpression of miRNAs strongly linked to the carcinogenesis just 24 hours after the exposure, while a significant down-regulation of the same miRNAs was observed seven days after the treatment [20].

Considering the fact that there is sparse of available data concerning the postexposure effect of N-methyl-N-nitrosourea on miRNA expression, and there is just little information can be available regarding the acute chemical carcinogenity, we investigated the expression level of let-7a, miR-21 and miR-146a after a single dose of MNU.

MATERIALS AND METHODS

6-8 week-old CBA/CA $H2^k$ haplotype mice, weighted 20 g, were divided into four groups, each composed of six male and six female animals. Two groups (group 1 and group 3) were intraperitoneally injected with 30 mg/kg body weight dose of MNU at the beginning of the experiment. The controls (group 2 and group 4) were fed by standard laboratory chew pellet and tap water ad libitum. Mice received human care and the experiment was carried out under the approval of the Institutional Revision Board. At 24 hours after the beginning of the investigation mice belonging to the groups 1 and 2 were autopsied. The mice belonging to groups 3 and 4 were autopsied one week after the start of the experiment. Liver, spleen, lungs and kidneys of the animals were removed during autopsy.

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. The isolated RNA was quantified by absorption photometry at 260/280 nm. Optical density of the RNA was between 1.9 and 2.1.

The miRNAs were reverse transcribed into cDNA by Transcriptor First Strand Synthesis Kit (Roche, Berlin, Germany). 2.0 µl the miRNA templates, 2.0 µl random hexamer primer, 9.0 µl H_2O, 4.0 µl transcriptor reverse transcriptase reaction buffer, 0.5 µl protector RNase inhibitor, 2.0 µl Deoxynucleotide Mix and 0.5 µl transcriptor reverse transcriptase were mixed and heated at 65 °C for 30 minutes.

Quantitative real-time PCR reactions were performed by LightCycler 480 system (Roche, Berlin, Germany) using LightCycler 480 SYBR Green I Master (Roche, Berlin, Germany). The reaction mix included: 5.0 µl cDNA template, 3.0 µl H_2O, 2.0 µl PCR primer and 10.0 µl master mix in 20 µl final volume. The reaction mixtures were incubated at 95 °C for 10 minutes, followed by 55 amplification cycles: denaturation at 95°C for 10 s, annealing at 50°C for 15 s, extension at 72°C for 20 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'-GTGCAGGGTCCGAGGT-3'; miR-21 forward: 5'-GCCCGCTAGCTTATCAGACTGATG-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'; miR-146a forward: 5'-GCCGCCCTGTGAAATTCAGTT-3', reverse: 5'-GTGCAGGGTCCGAGG -3'. The gene expression was determined by absolute nucleic acid quantification method with 4.0 Light Cycler software (Roche Diagnostics GmbH, Mannheim, Germany).

The expression levels of each miRNAs were determined by quantitative real-time PCR methods in 2011 and the statistical analysis was performed in 2012. Student's *t*-test was applied 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±2 SD. The calculation was performed using Statistical Program for Social Science 19.0 (SPSS) software (IBM, Armonk, New York, USA).

RESULTS

Using quantitative real- time PCR methods the expression profiles of miRNAs were evaluated and statistically analyzed (Table I) at 24th hour and one week after MNU administration.

As shown in Figure 1 A, the expression of the investigated miRNAs was strongly altered in liver tissues. At 24th hour after the MNU treatment there was a considerable down-regulation detectable in the expression of let-7a and miR-146a while miR-21 showed increased expression related to the control groups. Similar alteration tendency was observed one week after MNU exposure.

In the spleen the expression of all investigated miRNAs were found to be significantly increased by 1.93-10.47 fold change in both treated groups compared to the controls (Figure 1B). MiR-21 was the most strikingly upregulated miRNA. Furthermore, the magnitude of the changes in the expression level of miR-21 was higher at 24th hour timepoint than at the seventh day after the treatment.

Table 1. Results of statistical analysis.

	miRNA	*Fold change**	*p-Value*
Liver			
24 h	let-7a	0.05	$5.97 \cdot 10^{-3}$
	miR-21	3.58	$4.51 \cdot 10^{-4}$
	miR-146a	0.47	0.03
1 week	let-7a	0.45	0.07
	miR-21	4.07	$4.71 \cdot 10^{-5}$
	let-146a	0.94	0.91
Spleen			
24 h	let-7a	10.47	$5.07 \cdot 10^{-3}$
	miR-21	7.41	$4.65 \cdot 10^{-5}$
	miR-146a	1.93	$5.36 \cdot 10^{-6}$
1 week	let-7a	9.76	$5.20 \cdot 10^{-7}$
	miR-21	8.12	$2.98 \cdot 10^{-6}$
	miR-146a	2.00	$3.28 \cdot 10^{-5}$
Lungs			
24h	let-7a	1.87	0.02
	miR-21	0.87	0.55
	miR-146a	0.64	0.19
1 week	let-7a	1.49	0.15
	miR-21	2.25	$3.19 \cdot 10^{-3}$
	miR146a	0.09	$5.42 \cdot 10^{-5}$
Kidneys			
24 h	let-7a	0.59	0.02
	miR-21	2.81	$3.73 \cdot 10^{-5}$
	miR-146a	0.16	$1.10 \cdot 10^{-3}$
1 week	let-7a	3.34	$1.9 \cdot 10^{-4}$
	miR-21	2.86	$4.65 \cdot 10^{-6}$
	miR-146a	0.12	$8.26 \cdot 10^{-4}$

* The fold change values are the gene expression ratios of MNU treated mice over the control animals according to tissue samples, duration of the treatment and miRNAs.

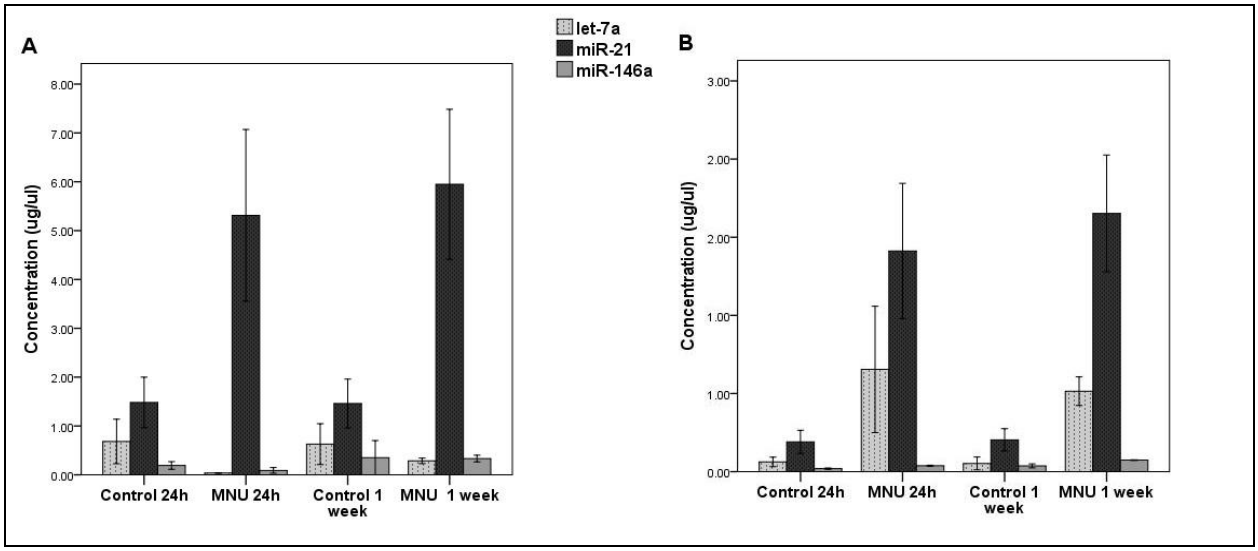

Figure 1. let-7a, miR-21, miR-146a gene expression in liver *(A)* and spleen *(B)* of mice at 24th hour and one week after MNU exposure.

There was no significant alteration detectable in the lungs at 24th hour after MNU treatment, while 7 days after the exposure we observed a significant increase in the expression of miR-21 and a reduction in miR-146 level compared to the controls (Figure 2A). The miR-146a gene expression in the treated group also showed markedly decrease in the kidney, where we found more than eight times lower level on the 7th day and six times lower level at the 24th hour timepoint in relation to the contols (Figure 2B).

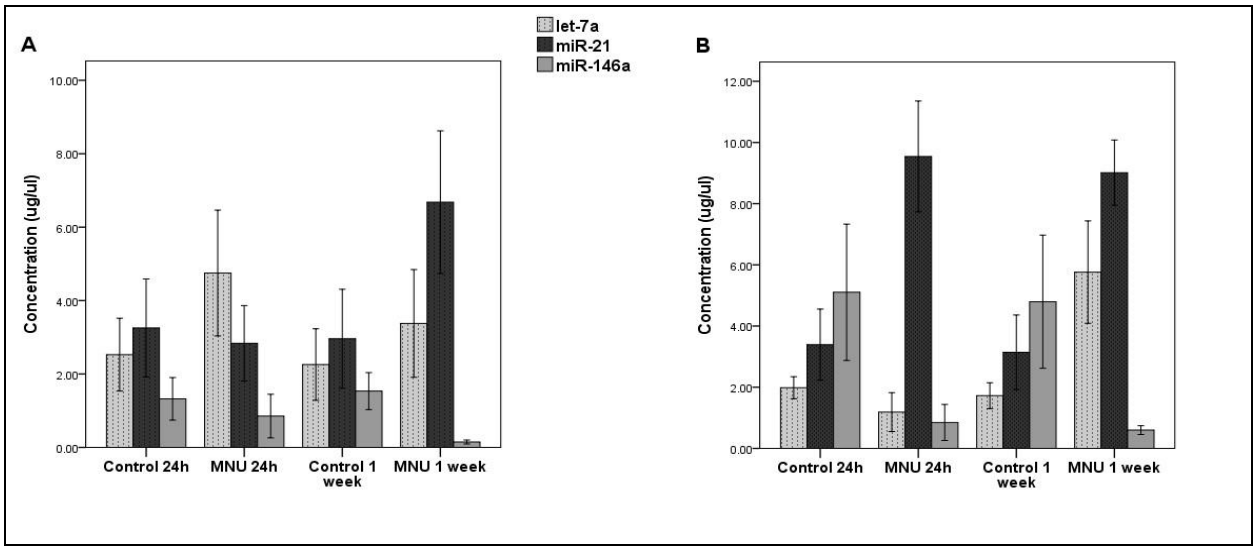

Figure 2. let-7a, miR-21, miR-146a gene expression in lung *(A)* and kidney *(B)* of mice at 24 hour and one week after MNU exposure.

DISCUSSION

Recent toxicological researches reported on the relation between toxicant exposure and alteration in miRNA expression profile [21]. It has also been demonstrated that the most of the dysregulated miRNAs by carcinogen agents are involved in gene regulation linked to mechanisms of tumorgenesis [22]. In the present study we aimed to evaluate the acute response of three different miRNAs expressions (let-7a, miR-21 and miR-146a) after single dose of MNU, a well-known direct-acting carcinogen.

Changes in miRNA expression profile induced by carcinogens may differ based on the type of the agent and the target tissue. Among many miRNAs miR-21 is frequently dysregulated in numerous types of human cancers [23-24]. Inhibiting the expression of several pro - apoptotic genes and tumor suppressor genes including p53, PTEN, PDCD4, TPM1, miR-21 contributes to genesis and progression of several types of tumors [25]. Number of studies investigated the miRNA responses to different chemical carcinogens, include 2-acetylaminofluorene, vinyl carbamate, hexahydro- 1, 3, 5- trinitro- 1, 3, 5- triazine, 7,12–dimethylbenz(α)anthracene reported on the upregulation of miR-21 [15, 17, 18, 19]. Although these studies determined the chronic and subchronic chemical carcinogenity of these agents, we also found an increased level of miR-21 just at 24th hour after the treatment in all investigated organs in correlation to the controls. The most markedly change was observed in spleen compared to the other organs. Additionally all of the miRNAs were overexpressed in the spleen, and the most strikingly changes in the miRNA expression levels were observed in the spleen. It can refer to the fact that MNU have a potential to induce tumorgenesis in the hematopoetic system [26]. When Chan et al. investigated the effect of N-ethyl-N-nitrosurea in mice spleen, their study gave parallel results. They found significantly higher level of miR-34a in spleen just 24th hour after N-ethyl-N-nitrosurea exposure [27].

In contrast to miR-21, the expression level of miR-146a, which is mainly involved in the regulation of NF-κB gene, showed a reduction after MNU treatment releated to the non-treated groups except of the spleen [28]. When Izotti et al. investigated rats, were exposed to environmental cigarette smoke for four weeks, showed a significant decrease of miR-146 level in lung tissues, while another study tested the miRNA responses to vinyl carbamate exposure in lung, revealed an elevated expression of miR-146 after in mice injected intraperitoneally with two doses of VC [12, 18].

Studies carried out with long exposure periods of environmental carcinogens such as cigarette smoke and RDX revealed the downregulation of let-7a, which seems to function as a tumour suppressor by regulating oncogens include RAS and c-myc genes [29, 12, 15]. We observed an opposite tendency of let-7a expression except of the liver after single injection of MNU.

Based on our results, the expression of the different miRNAs appear to be modified in response to various chemical agents and their target sites. The alterations we observed in let7-a, miR-21 and miR-146a expression induced by single dose of treatment can indicate their involvement in MNU induced carcinogenesis. The analysis of miRNA expression

pattern shows promise for explanation of the of chemically induced tumorgenesis and useful tool for detection of early stage of carcinogenesis.

ACKNOWLEDGEMENTS

This work was supported by NKTH, TECH -09-A4-2009-0133, BDREVAM2, Hungary.

REFERENCES

1. Allay E, Veigl M, Gerson SL. Mice ocer-expressing human O^6 alkyltransferase selectively reduce O^6 methylguanine madiated carcinogenic mutations to treshold levels after N-methyl-N-nitrosurea. Oncogene. 1999; 18: 3783-3787.
2. Kang HM, Jang JJ, Langford C, Shin SH, Park SY, Chung YJ. DNA copy number alterations and expression of relevant genes in mouse thymic lymphomas induced by gamma-irradiation and N-methyl-N-nitrosourea. Cancer Genet Cytogenet. 2006; 166(1): 27-35.
3. Lu J, Jiang C, Mitrenga T, Cutter G, Thompson HJ. Pathogenic characterization of 1-methyl-1-nitrosourea-induced mammary carcinomas in the rat. Carcinogenesis. 1998; 19(1): 223-227.
4. Mangues R, Kahn JM, Seidman I, Pellicer A. An overexpressed N-ras proto-oncogene cooperates with N-methylnitrosourea in mouse mammary carcinogenesis. Cancer Res. 1994; 54(24): 6395-6401.
5. Morton D, Bailey KL, Stout CL, Weaver RJ, White KA, Lorenzen MJ, Ball DJ. N-Methyl-N-Nitrosourea (MNU): A positive control chemical for p53+/- mouse carcinogenicity studies. Toxicol Pathol. 2008; 36(7*)*: 926-931.
6. Ando K, Saitoh A, Hino O, Takahashi R, Kimura M, Katsuki M. Chemically Induced Forestomach Papillomas in Transgenic Mice Carry Mutant Human c-Ha-ras Transgenes. Cancer Res. 1992; 52: 978-982.
7. Budán F, Varjas T, Nowrasteh G, Varga Z, Boncz I, Cseh J, Prantner I, Antal T, Pázsit E, Gobel G, Bauer M, Gracza T, Perjési P, Ember I, Gyöngyi Z. Early modification of c-myc, Ha-ras and p53 expressions by N-methyl-N-nitrosourea. In Vivo. 2008; 22(6): 793-797.
8. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116(2): 281-297.
9. Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Reviews Genetics. 2009; 10(10): 704-714.
10. Zhang B, Pan X, Cobb GP, Anderson TA. MicroRNAs as oncogenes and tumor suppressors. Dev Biol. 2007; 302(1): 1-12.
11. Lema C, Cunningham MJ. MicroRNAs and their implications in toxicological research. Toxicol Lett. 2010; 198(2): 100-105.
12. Izzotti A, Calin GA, Steele VE, Cartiglia C, Longobardi M, Croce CM, De Flora S. Chemoprevention of cigarette smoke-induced alterations of MicroRNA expression in rat lungs. *Cancer Prev Res.* 2010; 3(1): 62-72.
13. Li Z, Braham W, Dial S, Wang Y, Guo L, Shi L, Chen T. Genomic analysis of microRNA time-course expression in liver of mice treated with genotoxic carcinogen N-ethyl-N-nitrosurea. BMC Genomics. 2010; 11: 609.
14. Kalscheuer S, Zhang X, Zeng Y, Upadhyaya P. Differential expression of microRNAs in early-stage neoplastic transformation in the lungs of F344 rats chronically treated with the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Carcinogenesis. 2008; 29(12): 2394-2399.
15. Zhang B, Pan X. RDX induces aberrant expression of microRNAs in mouse brain and liver. Environ Health Perspect. 2009; 117(2): 231-240.
16. Pogribny IP, Tryndyak VP, Boyko A, Rodriguez-Juarez R, Beland FA and Kovalchuk O. Induction of microRNAome deregulation in rat liver by long-term tamoxifen exposure. Mutation Res. 2007; 619(1-2): 30-37.
17. Pogribny IP, Muskhelishvili L, Tryndyak VP, Beland FA. The tumor-promoting activity of 2-acetylaminofluorene is associated with disruption of the p53 signaling pathway and the balance between apoptosis and cell proliferation. Toxicology and Applied Pharmacology. 2009; 235: 305-311.
18. Melkamu T, Zhang X, Tan J, Zeng Y, Kassie F. Alteration of microRNA expression in vinyl carbamate-induced mouse lung tumors and modulation by the chemopreventive agent indole-3-carbinol. Carcinogenesis. 2010; 31(2): 252-258.
19. Yu T, Wang XY, Gong RG, Li A, Yang S, Cao YT, Wen YM, Wang CM, Yi XZ. The expression profile of microRNAs in a model of 7,12-dimethyl-benz[a]anthrance-induced oral carcinogenesis in Syrian hamster. J Exp Clin Cancer Res. 2009; 28(1): 64.
20. Juhász K, Gombos K, Szirmai M, Révész P, Magda I, Gőcze K, Ember I. DMBA Induces Deregulation of miRNA Expression of let-7, miR-21 and miR-146a in CBA/CA Mice. *In Vivo.* 2012; 26(1): 113-117.
21. Lema C, Cunningham MJ. MicroRNAs and their implications in toxicological research. Toxicol Lett. 2010; 198(2): 100-105.
22. Chen T. The role of MicroRNA in Chemical Carcinogenesis. Journal of Environmental Science and Health. 2010; 28: 89-124.
23. Liu C, Yu J, Yu S, Lavker RM, Cai L, Liu W, Yang K, He X, Chen S. MicroRNA-21 acts as an oncomir through multiple targets in human hepatocellular carcinoma. J Hepatol. 2010; 53(1): 98-107.
24. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer. 2005; 65: 6029-6033.
25. Zhu S, Wu F, Nie D, Sheng S, Mo YY. Micro-RNA-21

targets tumor suppressor genes in invasion and metastases. Cell Res. 2008; *18*: 350-9.

26. Long GG, Goodman DG, Credille KM, Mann PC, Wilson JM, Cardy R. Hematopoietic proliferative lesions in the spleen of rasH2 transgenic mice treated with MNU. Toxicol Pathol. 2010; 38(7): 1026-36.

27. Chen D, Li Z, Chen T. Increased expression of miR-34a in mouse spleen one day after exposure to N-ethyl-N-nitrosurea. J. Appl. Toxicol. 2011; 31(5): 496-498.

28. Zhao JL, Rao DS, Boldin MP, Taganov KD, O'Connell RM and Baltimore D: NF-{kappa}B dysregulation in microRNA-146a-deficient mice drives the development of myeloid malignancies. Proc Natl Acad Sci U S A. 2011; 108(22): 9184-9.

29. Akao Y, Nakagawa Y and Naoe T: *let-7* MicroRNA functions as a potential growth suppressor in human colon cancer cells. Biol Pharm Bull. 2006; 29(5): 903-6.