The Journal of Applied Pharmacology, **15**, 199-204(2007)

Genetic Toxicity Test of Methylcarbamate by Ames, Micronucleus, Comet Assays and Microarray Analysis

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(Received 18 July 2007; Accepted 14 September 2007)

Abstract – Carbamates have excellent insecticidal activities against a broad spectrum of insects. They possess knocking-down, fast-killing, and systemic effects, however, they are toxic to mammals. In this study, we have carried out in vitro genetic toxicity test of methylcarbamate and microarray analysis of differentially expressed genes in response to methylcarbamate. Methylcarbamate did not show mutations in base substitution strain TA1535 both with and without exogenous metabolic activation. Methylcarbamate did not show mutations in frame shift TA98 both with and without exogenous metabolic activation. Methylcarbamate showed DNA damage based on single cell gel/comet assay in L5178Y cells both with and without exogenous metabolic activation. Methylcarbamate did not increase micronuclei in CHO cells both with and without exogenous metabolic activation. Microarray analysis of gene expression profiles in L5178Y cells in response to methylcarbamate selected differentially expressed 132 genes that could be candidate biomarkers of genetic toxic action of methylcarbamate.

Keywords □ methylcarbamate, Ames test, COMET, MN assay, Microarray, S9 fraction

INTRODUCTION

Carbamates have excellent insecticidal activities against a broad spectrum of insects. They possess knocking-down, fast-killing, and systemic effects. However, they are toxic to mammals. Eya has reported that formamidine-S-carbamates possess improved ovicidal and acarcidal activities and are less toxic to mice than the methylcarbamates (Eya and Fukuto, 1986). Many biscarbamoyl sulfide derivatives of methylcarbamate insecticides have also been reported to retain the good insecticidal activity of the parent methylcarbamate but were substantially less toxic to the white mouse (Fahmy *et al.*, 1978). Benzoylphenylureas (BPUs), discovered in the 1970s, are known well as commercial chitin formation inhibitors. In contrast to traditional pesticides, BPU and its derivatives mainly control the growth and development process of insects by interfering with chitin biosynthesis and breeding (Van Daalen et al., 1972; Post and Vicent, 1973; Verloop and Ferrel, 1977). Consequently, the toxicity of BPUs to vertebrates and environmental impact is very low and a high insecticidal selectivity is achieved. However, BPUs do not have systemic properties (Mass *et al*., 1981). As a consequence, BPUs cannot effectively control sucking pests such as aphids and hidden feeders such as bollworms, budworms, and stem borers. The toxicity of organophosphate (OP) and carbamate (CB) insecticides is caused by a progressive inhibition of AChE in neural tissue, resulting in the accumulation of ACh in the synaptic cleft (Yi *et al*., 2006) Subsequently, the over stimulation of ACh receptors leads to neurotoxic symptoms (Yi *et al.*, 2006). Synthetic carbamate insecticides have been widely used in China in the last two decades. The carbamate insecticides have caused serious threat to non-target organisms, including fish, in the environment due to the overuse of these compounds. Methomyl, thiodicarb, carbofuran and carbosulfan are four major carbamate insecticides that have been widely used for control of insect pests. Thiodicarb and carbosulfan are derivates of methomyl and carbofuran by structural modification resulting in decreased toxicity to mammals (Duan *et al*., 1998 and Zeng *et al*., 1995).

Several genetic toxicity assays systems were developed to measure DNA damage and mutagenicity, however, for safety assessment of chemicals combination of test needed because no single test was capable of detecting all genotoxic chemicals.

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In this study, we have tested methylcarbamate for genetic toxicity using Ames test, in vitro micronuclei assay in CHO cells, single cell gel/comet assay in L5178Y cells. And microarray analysis of gene expression profiles in L5178Y cells in order to find out biomarker gene expression profile of genetic toxicity of methylcarbamate.

MATERIALS AND METHODS

Materials

Methylcarbamate, 2-aminofluorene, 2-nitrofluorene, sodium azide, methane-sulfonic acid methyl ester, benzo(a)pyrene and cyclophosphamide were obtained from Sigma chemical Co. (St. Louis, MO). The S9 fraction was purchased from Moltox® S9 (Canbiotech).

Ames test

The Ames test was performed by the pre-incubation test method (Gatehouse et al., 1994) with or without metabolic activation using Salmonella typhimurium strains TA98 and TA1535. The tester strains were cultured overnight in nutrient broth medium at 37°C. To the 0.1 ml of bacterial suspension, 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) or 0.5 ml of S9 was added and then 0.1 ml of methylcarbamate (10, 33.3, 100, 333.3, 1000 μg/plate) or positive control chemicals such as 2-aminofluorene, 2-nitrofluorene and sodium azide were added and incubated for 20 min at 37°C. After incubation, 2.0 ml of top agar was added to mix and the mixture was poured onto a minimal glucose agar plate. 48 Hours after the incubation at 37°C, the numbers of revertant colonies were counted (Kasamatsu et al., 2005).

Comet assay

Comet assay was carried out according to Singh et al. (Singh *et al.*, 1988) with slight modification. L5178Y mouse lymphoma cells were grown at 37°C in a 5% CO_2 incubation. L5178Y mouse lymphoma cells were seeded in 12 well plates ($1X10^6$ cells/ml) and were exposed to 1250, 2500, 5000 μg/ml methylcarbamate for 2 h. Positive controls were 150 μM methyl methanesulfonate (MMS) in the absence of S9, 50 μM benzo(a)pyrene (BaP) in the presence of S9 metabolic activation. 20 μl of cell suspension were mounted in 1% agarose on slide glass. Slides were immersed in a cold lysing solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1% (v/v) Triton X-100 and 10% (v/v) DMSO) for 1.5h at 4°C and then for 20min in the electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH >13). Slides were electrophoresed and neutralized using Tris buffer (0.4 M Tris, pH 7.5) and stained with ethidium bromide (20 μg/ml). Cells were analyzed using a Comet Image Analysis System, Version 5.5 (Kinetic Imaging Ltd., Andor Bioimaging Division, Nottingham, UK).

In vitro cytokinesis block micronucleus assay

The CBMN (cytokinesis-block micronucleus) assay was performed according to Fenech (Fenech., 2000) with modification, and the recommendation of the 3rd International Workshop on Genotoxicity Testing (Kirsch-Volders *et al.*, 2003). CHO-K1 cells were grown in 24-well plates and treated with methylcarbamate (1250, 2500, 5000 μg/ml) or cyclophosphamide (2.5, 5, 10 μg/ml) for 4 h with or without S9. After the treatment, cells were washed with PBS and further incubated for 20 h in the medium containing 3 μg/ml cytochalasin B. Cells were harvested and spread on glass slide, and fixed with 100% methanol for 5 min and stained with 0.24 mM acridine orange in 1/150 M phosphate buffer (pH 6.8) for 3 min. Micronuclei were scored under the fluorescence microscope at 1000 magnification.

Microarray

The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification. L5178Y mouse lymphoma cells were plated in RPMI-1640 medium into 12-well plate. After 2 h of treatment with methylcarbamate (5000 μg/ml), cells were resuspended in media without methylcarbamate and cultured for 20 h. Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) and purified by a RNeasy mini kit (QIAGEN, Hilden, Germany). Total RNA (1 μg) was amplified using the Affymetrix one-cycle cDNA synthesis protocol. For each array, 15 μg of amplified biotin-cRNAs was fragmented and hybridized to the Affymetrix Mouse Genome 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA) for 16 h at 45°C in a rotating hybridization oven. Slides were stained with streptavidin/phycoerythrin and washed for antibody amplification. Arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037).

RESULTS

Methylcarbamate induced gene mutations in both TA98 and TA1535 strains

The mutant frequency (MF) was assessed as a measure of gene mutation in both TA98 and TA1535 strains exposed to

different concentrations of methylcarbamate (Fig. 1). In TA98 strain, the MF of 1.0 μg/plate 2-nitrofluorene treated bacteria in the absence of S9 was 149.67±17.10 and the MF of cells exposed to 10 μg/plate 2-aminofluorene in the presence of S9 was 186.67± 10.02. The positive control chemicals, 2-nitrofluorene and 2-aminofluorene generated large increases in number of revertants. The MF of solvent control bacteria were 28.00±2.00 in the absence of S9 and 33.67±5.03 in the presence of S9. The MF of methylcarbamate (10, 33.3, 100, 333.3, 1000 μg/plate) treated bacteria were 35.33±3.79, 36.00±2.00, 34.67±5.13, 27.67± 5.69, 29.33±2.08 in the absence of S9, 33.00±7.81, 39.00±4.36, 35.67±17.21, 35.67±11.24, 42.33± 4.73 in the presence of S9, respectively. Methylcarbamate treatments did not increase in revertant numbers in TA98 with or without S9. In TA1535 strain, the MF of 1.5 μg/plate sodium azide treated cells in the absence of S9 was 283.00±24.98 and the MF of bacteria exposed to 10 μg/plate 2-aminofluorene in the presence of S9 was 273.33±3.06. The positive control chemicals, sodium azide and 2-aminofluorene resulted large increases in revertant numbers. The MF of solvent control bacteria were 24.67±4.51 in the absence of S9 and 29.00±6.24 in the presence of S9. The MF of methylcarbamate (10, 33.3, 100, 333.3, 1000 μg/plate) treated bacteria were 81.00±7.81, 87.00± 1.00, 86.67±3.51, 82.00±8.19, 88.00±7.00 in the absence of S9, 51.00±9.54, 52.33±9.29, 47.33±4.73, 40.33±4.93, 48.00±7.81 in the presence of S9, respectively. Methylcarbamate treatments statistically significant increased in revertant numbers in TA1535 with or without S9. However, methylcarbamate did not show the dose-dependent increases. Thus, methylcarbamate showed negative response in Ames test using both TA98 and TA1535.

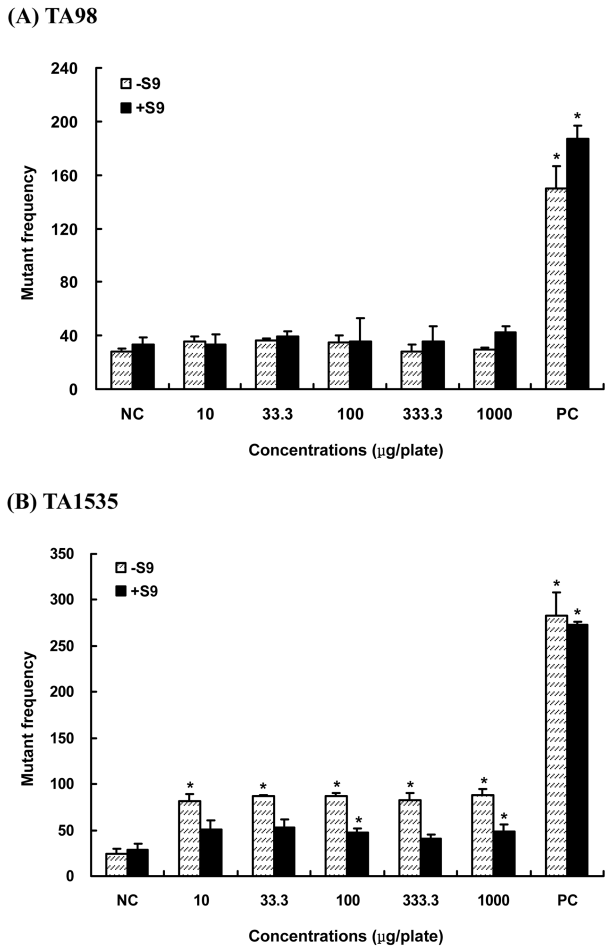

Fig. 1. The mutagenicity of Methylcarbamate tested in strain TA98 and TA1535. The Ames test was performed by the pre-incubation test method (Gatehouse et al., 1994) with or without metabolic activation using Salmonella *typhimurium strains* TA98 and TA1535 as described in methods. The data represent averages from three experiments with triplicate plates per dose. NC: negative control. PC: positive control (-S9: 1.0 μg/plate 2-nitrofluorene, 1.5 μg/plate sodium azide, +S9: 10 μg/plate 2-aminofluorene)

Methylcarbamate induced DNA damage in L5178Y cells

The Olive Tail Moment (OTM) was assessed as a measure of DNA damage in the comet assay in L5178Y mouse lym-

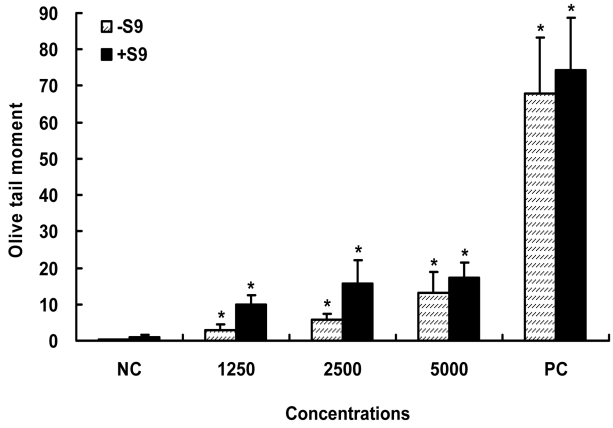

Fig. 2. Olive tail moments by Methylcarbamate in L5178Y mouse lymphoma cells. Olive tail moments were measured using comet assay according to Singh et al. (Singh et al., 1988) with slight modification as described in methods. Olive tail moments of L5178Y mouse lymphoma cells exposed to 1250, 2500, 5000 μg/ml methylcarbamate for 2h. Negative control was medium. Positive controls were MMS (150 μM) in the absence of S9 and BaP (50 μM) in the presence of S9 metabolic activation system, respectively. NC: negative control. PC: positive control, Data are means ± S.D. (n=15)

phoma cells exposed to different concentrations of methylcarbamate (1250-5000 μg/ml) for 2 h (Fig. 2). The OTM of MMS-treated cells (150 μM, positive control in the absence of S9) was 67.98±15.21 and the OTM of cells exposed to B[a]P (50 μM, positive control in the presence of S9 metabolic activation system) was 74.36±14.20. The OTM of control cells was 0.22±0.25 in the absence of S9 and 0.86±0.67 in the presence of S9. Cells were exposed to 1250, 2500, 5000 μg/ml methylcarbamate for 2 h. OTMs induced by methylcarbamate were 2.90±1.48, 5.61±1.65, 13.12±5.82 in the absence of S9 and 9.94±2.69, 15.82±6.21, 17.14±4.35 in the presence of S9, respectively. It thus caused a significant increase in DNA damage in comparison to the solvent control.

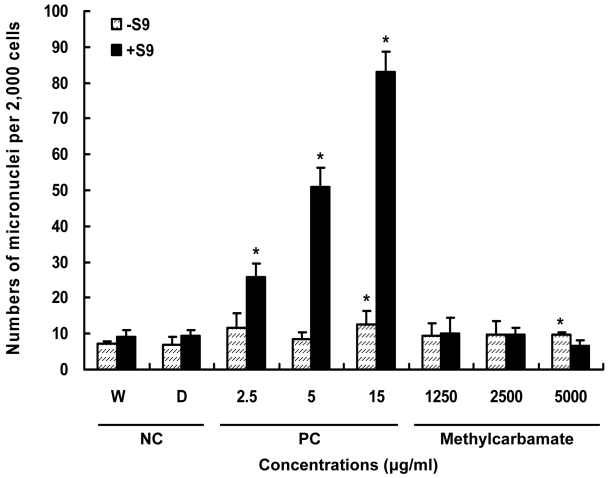

Fig. 3. Micronucleus formation by Methylcarbamate in CHO-K1 cells. The CBMN (cytokinesis-block micronucleus) assay was performed according to Fenech (Fenech, 2000) with modification, and the recommendation of the 3rd International Workshop on Genotoxicity Testing (Kirsch-Volders et al., 2003) as described in methods. CHO-K1 cells were grown in 24-well plates and treated with methylcarbamate (1250, 2500, 5000 μg/ml) or cyclophosphamide (2.5, 5, 15 μg/ml) for 4 h with or without S9. Cells were stained with 0.24 mM acridine orange and micronuclei were scored under the fluorescence microscope at 1000 magnification. Data are means ± S.D. n=3 *: Statistically different from concurrent control at $p<0.05$. NC: negative control. PC: positive control (cyclophosphmide 2.5, 5 and 15 μg/ml).

Methylcarbamate induced micronuclei in CHO-K1 cells

The MF of solvent control H_2O, DMSO were 7.33±0.58, 7.00±2.00 in the absence of S9 and 9.00±2.00, 9.33±1.53 in the presence of S9 CHO-K1 cell cultured RPMI medium and treated with cyclophosphamide(CPA) in the presence of S9. As expected, numbers of micronuclei were induced to be 2.5, 5, 15 μg/ml CPA 11.67±4.16, 8.33±2.08, 12.67±3.79, respectively. Cells were exposed 1250, 2500, 5000 μg/ml methylcarbamate for 4 h. Numbers of micronuclei of methylcarbamate treated cells were 9.33±3.51, 9.67±3.79, 9.67±0.58 in the absence of S9 and 10.00±4.58, 9.67±2.08, 6.67±1.53 in the presence of S9, respectively. Increase in the numbers of micronuclei with methylcarbamate treatment was not observed (Fig. 3).

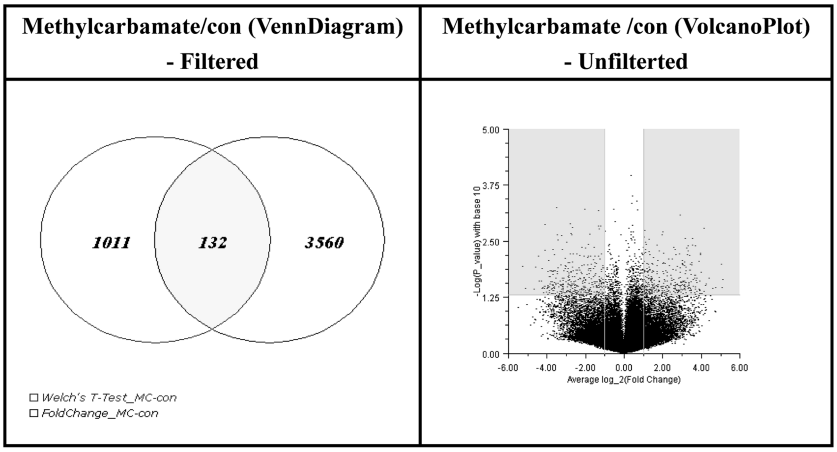

Fig. 4. Numbers of Methylcarbamate regulated genes in L5178Y cells. The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification as described in methods. L5178Y mouse lymphoma cells were treated with methylcarbamate (5000 μg/ml), and total RNA was isolated by TRIzol. After the hybridization and staining arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037). Profiles were analyzed by VennDiagram (A) and Volcano Plot (B).

Microarray analysis of differentially expressed genes with methylcarbamate treatment in L5178Y cells

Differentially expressed genes from L5178Y cells treated with methylcarbamate (5000 μg/ml) were analyzed by microarray using Affymetrix Mouse Genome 430 2.0 GeneChip arrays. 1143 genes were methylcarbamate specifically regulated and their fold of change were greater than Log 2. Among them 132 genes were selected after the Welch's T-test and Volcano plot analysis (Fig. 4). Figure 5 showed the results of clustering analysis of methylcarbamate regulated genes. Table I showed genes which expression was increased with methylcarbamate treatment. If these genes expression would be related to genetic toxicity of methylcarbamate, it would need further study.

DISCUSSION

The pollution of aquatic systems by pesticides, such as insecticides, which are often used in agriculture to reduce or destroy pests (Wang and Freemark, 1995; Van der Brink and Ter Braak, 1999), has attracted great concerns from the public. Since the first successful carbamate insecticide, carbaryl, was introduced in 1956, more carbamate insecticides (their mode of action is that of inhibiting the vital enzyme cholinesterase) have been used worldwide than many of the remaining others combined. Two distinct qualities have made carbaryl the most popular carbamate: its very low mammalian oral and dermal toxicity and its exceptionally broad spectrum of insect control (Ware, 2001). However, it is easily washed off the leaves and soil, may enter freshwater ecosystems by spray drift, leaching, runoff, or accidental spills, and presents potential risks for aquatic flora. Alterations of the species composition of an aquatic community as a result of toxic stress may affect the structure and the functioning of the whole ecosystem (Campanella *et al*., 2000; Wong, 2000; Verdisson *et al.*, 2001). According to the magnitude of the potential ecosystem risk, the decreasing order of the ecosystem risk was carbosulfan > propoxur > carbofuran > carbaryl, metolcarb. There was a strong variance between toxicity and ecosystem risk; i.e., "low toxicity" does not always imply "low ecosystem risk." Single-species toxicity tests have historically been the sources of biological data for hazard evaluation; however, whether information from these tests alone is suitable for predicting effects at the ecosystem level is a subject of discussion (Cairns *et al.*, 1996; Sanchez and Tarazona, 2002). Furthermore, multiple-species toxicity tests enable observation of the indirect effects of chemicals caused by interactions among species.

The result of this study shows the 132 genes induced or

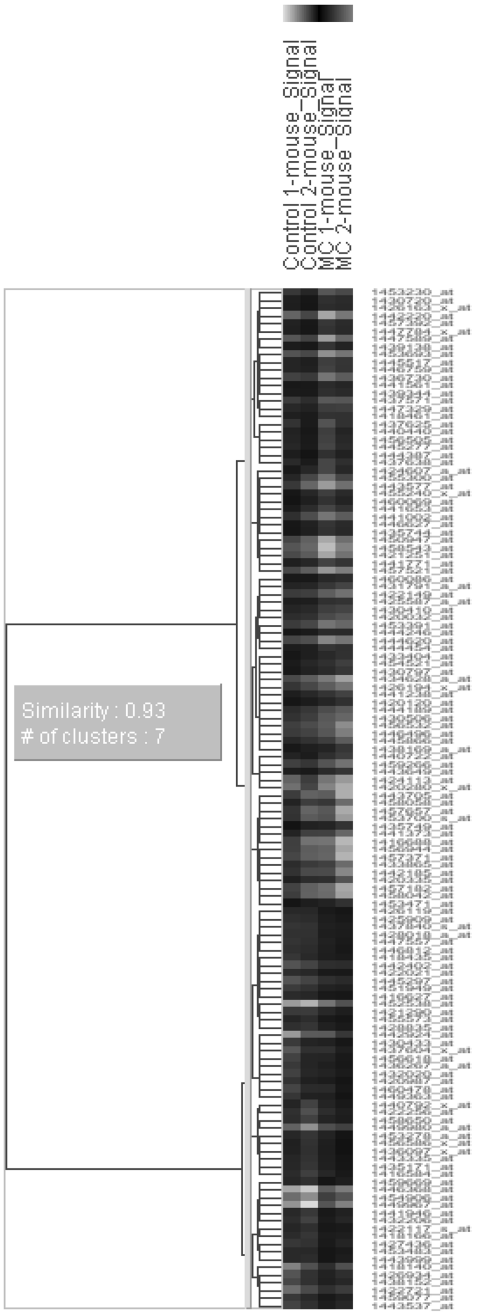

Fig. 5. Results of hierarchical clustering of genes regulated by Methylcarbamate. The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification as described in methods. L5178Y mouse lymphoma cells were treated with Methylcarbamate (5000 μg/ml), and total RNA was isolated by TRIzol. After the hybridization and staining arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037). The data were analyzed by hierarchical clustering, and green represents down regulation of the transcripts; black, no change; red, up regulation of the transcript.

Table I. Results of gene ontology analysis.

Accession No	Gene symol	Gene description	Fold change
NM_011376	Sim1	single-minded homolog 1 (Drosophila)	34.91
NM_011243	Rarb	retinoic acid receptor, beta	11.01
NM_008072	Gabrd	gamma-aminobutyric acid (GABA-A) receptor, subunit delta	10.28
NM_010025	Dcx	doublecortin	7.54
NM_031880	Tnk1	tyrosine kinase, non-receptor, 1	5.65
NM_021506	Sh3rf1	SH3 domain containing ring finger 1	4.17
NM_173390	Nhsl1	NHS-like 1	3.51
NM_133237	Apcdd1	adenomatosis polyposis coli down-regulated 1	3.51
NM_001042606	Sstr2	somatostatin receptor 2	3.31
NM_010331	Gpaa1	GPI anchor attachment protein 1	3.04

repressed from the methylcabamate treated cells. However, it should be pointed out that additional experiments (e.g., real-time RT-PCR) may be necessary to further confirm the utility of these three gene changes as biomarkers of exposure to methylcabamate that induce DNA damage through the above mechanism.

ACKNOWLEDGMENTS

This work was supported by grant 06132NTP396 from the KFDA of Korea.

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