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# Assessment of global and gene-specific DNA methylation in rat liver and kidney in response to non-genotoxic carcinogen exposure

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ABSTRACT

Altered expression of tumor suppressor genes and oncogenes, which is regulated in part at the level of DNA methylation, is an important event involved in non-genotoxic carcinogenesis. This may serve as a marker for early detection of non-genotoxic carcinogens. Therefore, we evaluated the effects of non-genotoxic hepatocarcinogens, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), hexachlorobenzene (HCB), methapyrilene (MPY) and male rat kidney carcinogens, d-limonene, p-dichlorobenzene (DCB), chloroform and ochratoxin A (OTA) on global and CpG island promoter methylation in their respective target tissues in rats. No significant dose-related effects on global DNA hypomethylation were observed in tissues of rats compared to vehicle controls using LC–MS/MS in response to short-term non-genotoxic carcinogen exposure. Initial experiments investigating gene-specific methylation using methylation-specific PCR and bisulfite sequencing, revealed partial methylation of *p16* in the liver of rats treated with HCB and TCDD. However, no treatment related effects on the methylation status of *Cx32*, *e-cadherin*, *VHL*, *c-myc*, *Igfbp2*, and *p15* were observed. We therefore applied genome-wide DNA methylation analysis using methylated DNA immunoprecipitation combined with microarrays to identify alterations in gene-specific methylation. Under the conditions of our study, some genes were differentially methylated in response to MPY and TCDD, whereas d-limonene, DCB and chloroform did not induce any methylation changes. 90-day OTA treatment revealed enrichment of several categories of genes important in protein kinase activity and mTOR cell signaling process which are related to OTA nephrocarcinogenicity.
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## 1. Introduction

Carcinogens are generally considered to increase the risk of cancer by either genotoxic or non-genotoxic (epigenetic) mechanisms. Genotoxic carcinogens or their metabolites directly interact with genomic DNA, leading to mutations in target cells (Klaunig et al., 2000), whereas non-genotoxic carcinogens show no DNA reactivity and act by alternative mechanisms. In contrast to genotoxic carcinogens, for which a variety of standard in vitro and in vivo genotoxicity assays are available, there are limited short-term tests which can be used to predict carcinogenicity of compounds operating by a non-genotoxic mode of action (Watson et al., 2004) and so far the only means to reliably detect carcinogenic potential remains the chronic rodent carcinogenicity bioassay. There is a strong interest in the detection of suitable biomarkers and development of short-term tests for early detection of non-genotoxic carcinogens (Moggs et al., 2004; Koturbash et al., 2011; LeBaron et al., 2010; Pogribny and Beland, 2013; Ziech et al., 2010).

Non-genotoxic carcinogens are thought to induce tumors by disturbing the balance between cell growth and death through interference with molecules involved in the regulation of both processes. Increased cell proliferation, evasion of apoptosis, disruption of gap

*Abbreviations:* 5-mdC, 5-methyldeoxycytidine; BSP, bisulfite sequencing; *Cx32*, *connexin-32*; DCB, dichlorobenzene; 2-dG, 2-deoxyguanosine; DNMT, DNA methyltransferases; *Cdh1*, *e-cadherin*; *cpne4*, *copine 4*; GO, gene ontology; HCB, hexachlorobenzene; *Igfbp2*, *insulin-like growth factor binding protein 2*; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC–MS/MS, liquid chromatography/tandem mass spectrometry; *LINE*, *long interspersed nucleotide elements*; MeDIP, methylated DNA immunoprecipitation; MPY, methapyrilene; MSP, methylation-specific PCR; OTA, ochratoxin A; *p15*, *Cdkn2b*; *p16*, *Cdkn2a*; PPI, protein-protein interaction; qPCR, Real-Time quantitative PCR; *SINE*, *short interspersed nucleotide elements*; *Tsc2*, *tuberous sclerosis 2*; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; *VHL*, *von Hippel-Lindau*.

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junction mediated growth control, oxidative stress and altered expression of tumor suppressors or oncogenes have been identified as early events in non-genotoxic carcinogenesis (Klaunig et al., 2000). Several groups investigated the potential of toxicogenomics approaches to classify genotoxic and non-genotoxic carcinogens and reported that gene expression signatures may be predictive of non-genotoxic carcinogenicity in rat liver (Ellinger-Ziegelbauer et al., 2008; Uehara et al., 2008a, 2011; Yamada et al., 2013; Römer et al., 2014; Eichner et al., 2014).

DNA methylation is an important epigenetic mechanism that affects cell function by altering gene expression. DNA methylation refers to the covalent addition of a methyl group, catalyzed by *DNA methyltransferases* (*DNMT*), to cytosine at carbon #5 to form 5-methylcytosine (Das and Singal, 2004). Approximately 2–5% of all cytosine residues in the mammalian genome are methylated and base-pair with guanine (Bombail et al., 2004). Altered DNA methylation may contribute to carcinogenesis in several ways, including global genomic DNA hypomethylation, hypomethylation of individual genes and tumor suppressor gene silencing through hypermethylation of CpG islands of genes (Watson and Goodman, 2002). Tumor cells frequently show a combination of global hypomethylation and gene-promoter specific hypermethylation (Bombail et al., 2004).

DNA methylation changes have been reported to occur early in carcinogenesis (Laird, 1997). It is also speculated that altered DNA methylation is a mechanism involved in multiple types of chemical-induced toxicities (Watson and Goodman, 2002) and may be a key feature in the mode of action of some non-genotoxic carcinogens (Bombail et al., 2004; Szyf, 2011). For example, studies by Counts et al. (1996) show that a tumor-inducing dose of the non-genotoxic rodent hepatocarcinogen phenobarbital induces global hypomethylation in the tumor-sensitive mouse strain B6C3F1. Pereira et al. (2004) described DNA hypomethylation in response to a number of non-genotoxic carcinogens, including bile acids, rutin, bromdichloromethane and 5-aza-2-deoxycytidine in mouse and/or rat colon. Coffin et al. (2000) reported that trihalomethane chloroform, bromodichloromethane, chlorodibromomethane and bromoform were more effective in causing DNA hypomethylation (e.g. of *c-myc*) when administrated by gavage than in drinking water in mouse liver, which correlates with their carcinogenic activity. Additionally, oral administration of Wy-14,643 (50 mg/kg bw, daily), a peroxisome proliferator and rodent hepatocarcinogen was shown to induce hypomethylation of c-myc gene at 48, 72 and 96 h after the first dose in mouse livers (Ge et al., 2001). In vivo 4-week phenobarbital-treatment study showed that phenobarbital caused alterations in DNA methylation in the liver and kidney tissues using MeDIP-coupled microarray profiling (Lempiäinen et al., 2011). Collectively, these studies suggest that alterations in DNA methylation may be an early event associated with the action of non-genotoxic carcinogens and may serve as an early marker for the prediction of carcinogenic potential.

Thus, the aim of this study was to investigate the dose-related effects of a range of non-genotoxic carcinogens on global and CpG promoter DNA methylation to determine if aberrant DNA methylation is an early and unifying feature of non-genotoxic carcinogenesis operating through different primary mechanisms. Archived tissue samples from previous experiments in which rats were treated with the non-genotoxic hepatocarcinogens 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), hexachlorobenzene (HCB), methapyrilene (MPY) and the male rat kidney carcinogens, d-limonene, *p*-dichlorobenzene (DCB) and chloroform for 28 days and ochratoxin A (OTA) for up to 90 days were analyzed for changes in DNA methylation.

Specifically, the work involved determination of global DNA methylation by liquid chromatography/tandem mass spectrometry (LC–MS/MS) and investigation of altered CpG DNA methylation in promotor regions of the proto-oncogene *c-myc* and several key tumor-suppressor genes involved in growth control, including *Cdkn2a* (*p16*), *Cdkn2b* (*p15*), *Cdh1* (*e-cadherin*), *connexin-32* (*Cx32*), *insulin-like growth factor binding protein 2* (*Igfbp2*) and *von Hippel-Lindau* (*VHL*) using methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing (BSP). Since non-genotoxic carcinogens influence the expression of genes involved in cell cycle, cell proliferation and apoptosis (e.g. *c-myc*, *p16*, *p15*, *e-cadherin*, *igfbp2*), we selected a number of such genes that might be modulated by DNA methylation. Furthermore, for the determination of genome-wide methylation we used the "open" unbiased method of methylated DNA immunoprecipitation (MeDIP) combined with microarray analysis to find out whether altered DNA methylation may provide a source for new biomarkers for early detection of non-genotoxic carcinogens. Several genes were affected by the changes in the methylation levels of their promoters. We used DAVID and PANOGA annotation tools to analyze these data further in a pathway related context to shed light into the mechanism of non-genotoxic carcinogenesis.

## 2. Material and methods

### 2.1. Origin of tissue samples

Archived tissue samples were obtained from our previous studies described in detail elsewhere (Mally and Chipman, 2002; Rached et al., 2007). Briefly, F344 rats (6–7 weeks old, n = 3–5 per group) were

**Table 1**
Primers used MSP, BSP and qPCR analysis and the corresponding annealing temperatures.

| | Gene name | | Sense (5′-3′) | Antisense (5′-3′) | PCR length (bp) | Ta (°C) | Reference |
|---|---|---|---|---|---|---|---|
| MSP | *e-cadherin* | M | GTTTTTAGTTAATTAGCGGCGTC | AAACTCAATAATACGCCGCG | 107 | 60 | Niwa et al. (2005) |
| | | U | GTGTTTTTAGTTAATTAGTGGTGTT | CTTAAAAACTCAATAATACACCACA | 114 | 57 | |
| | *p16* | M | AATTCGAGGAGAGCGATTCG | AACGTTTAATAAAACCCCGA | 123 | 55 | Swafford et al. (1997) |
| | | U | GTGAATTTGAGGAGAGTGATTTG | CAAAACATTTAATAAAACCCCAA | 123 | 55 | |
| | *VHL* | M | GTACGTTCGCGTCGTTTAC | TTCACAAAACGTAAAACCGA | 154 | 55 | Methyl Primer Express® (Applied Biosystems, |
| | | U | GTATGTTTGTGTTGTTTAT | TTCACAAAACATAAAACCAA | 154 | 51 | Foster City, CA, USA) |
| | *c-myc* | M | TTTGTTTTTTCGATTTTAGAGAGAC | TTATCCTACGTATATTAATCACCGC | 126 | 55 | Meth-Primer Design (Li and Dahiya, 2002) |
| | | U | TGTTTTTTTGATTTTAGAGAGATGA | CTTATCCTACATATATTAATCACCACA | 127 | 55 | |
| | *Igfbp2* | M | TTTCGGGTGAGAAAAGATC | AACCGAAATACCGAAATACAA | 147 | 51 | Methyl Primer Express® (Applied Biosystems, |
| | | U | AGATTTTGGGTGAGAAAAGATT | AACCAAAATACCAAAATACAACTA | 147 | 55 | Foster City, CA, USA) |
| | *p15* | M | CGAGAGGGTTTTTTGGATAC | AACGTACAAATACCTCGCAA | 138 | 51 | Methyl Primer Express® (Applied Biosystems, |
| | | U | GTTTGAGAGGGTTTTTTGGATAT | AACATACAAATACCTCACAA | 138 | 55 | Foster City, CA, USA) |
| | *Cx32* | M | AATTTAGATAGTTCGGTTATTTCGG | ACAACATATAACTCTCCAACACCG | 155 | 60 | Methyl Primer Express® (Applied Biosystems, |
| | | U | AATTTAGATAGTTTGGTTATTTTGG | AACAACATATAACTCTCCAACACCA | 156 | 55 | Foster City, CA, USA) |
| BSP | *p16* | | GTTTGTTGGGAGGAGGAGAGATT | ACTAATCTATCTACAAAAAACTCAT | 505 | 55 | Abe et al. (2002) |
| | *c-myc* | | ATTTGTTTTTAGTGGAAATTGGTTG | TACTACCAAAAAAACCCTCCACTC | 241 | 55 | Chen et al. (2010) |
| qPCR | *h19* | | CTCGGGTAACTCCTTCGGTCTTG | GCGGCATATTTGTCCATCTAGC | 93 | 60 | Chen et al. (2010) |
| | *c-myc* | | CAGAGACTCGACCGGGAGGATC | CTCCTTGCCCCGTAGGTCTTTC | 100 | 60 | Chen et al. (2010) |
| | *ID* | | TGGCAGCAAGAGCTAACGTTCGT | TTCGGAGCTGAGGACCGAA | 98 | 60 | Chen et al. (2010) |
| | *LINE-1* | | CGTTCTGTGTTTACCGGAAGTC | TTGGTGGTCTCTTCCGCTCT | 139 | 60 | Chen et al. (2010) |

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treated with the non-genotoxic hepatocarcinogens TCDD, HCB, MPY and the male rat kidney carcinogens, d-limonene, DCB, and chloroform for 28 days (Mally and Chipman, 2002) and with OTA for up to 90 days (Rached et al., 2007). The dosing regimen for each compound was based on the respective cancer bioassay. TCDD (0; 0.0025; 0.025; 0.25 μg/kg, bw) was dissolved in corn oil and administered to female F344 rats by gavage (2 days/week) for 28 days (NTP, 1982). MPY (0, 40, 200, 1000 ppm) and HCB (0, 8, 40 200 ppm, dissolved in 2% arachis oil) were added to powdered diet and fed to female F344 rats for 28 days ad libitum (Ertürk et al., 1985; Lijinsky et al., 1980). d-Limonene (0, 15, 75, 150 mg/kg bw), DCB (0, 30, 150, 300 mg/kg bw) and chloroform (0, 18, 90, 180 mg/kg bw) were dissolved in corn oil and administered by gavage (5 days/week) for 28 days (NCI, 1976; NTP, 1987; NTP, 1990). OTA (0, 21, 70, 210 μg/kg, bw) was dissolved in corn oil and administered by gavage (5 days/week) for 14, 28, and 90 days (NTP, 1989). Control animals received vehicle only. At the end of each treatment, rats were killed by $CO_2$ asphyxiation and cervical dislocation, the livers and kidneys were removed, snap frozen in liquid nitrogen and stored at −70 °C until analysis.

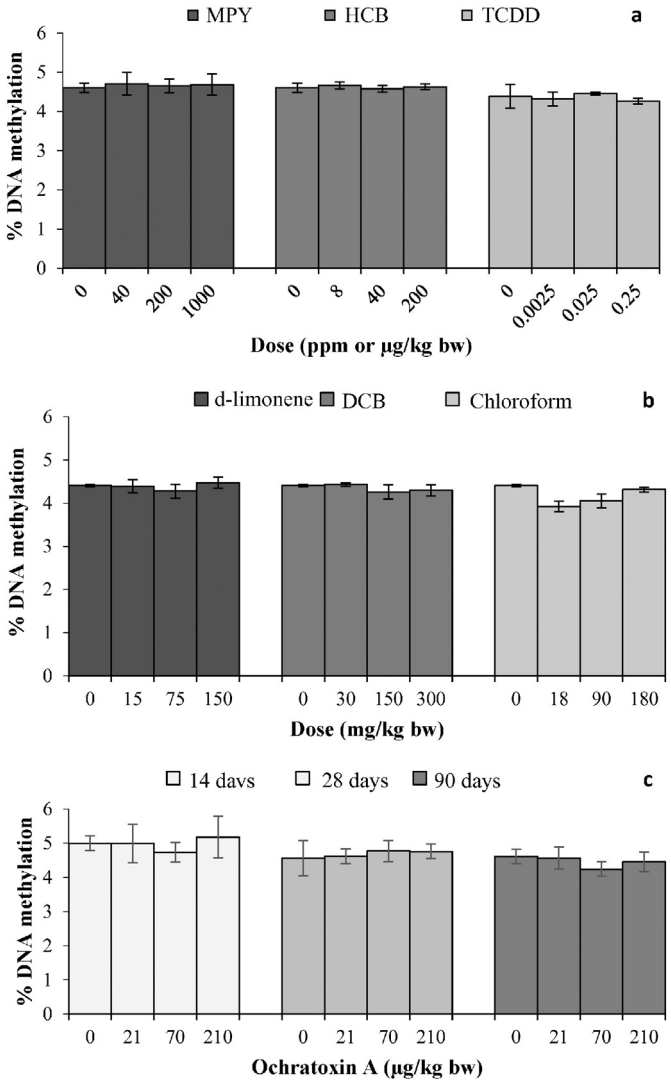



**Fig. 1.** Effects of non-genotoxic carcinogens on global DNA methylation levels in a) livers of female rats treated with non-genotoxic hepatocarcinogens for 28 days, b) kidneys of male rats treated with male rat carcinogens for 28 days and c) kidneys of male rats treated with OTA for 14, 28, and 90 days. Values represent mean ± SD (n = 3–5).

### 2.2. Isolation of DNA from liver and kidney tissues

Genomic DNA was isolated from 40 to 100 mg of male rat liver and kidney tissues using NucleoBond® AXG 100 columns and the NucleoBond® Buffer Set IV (Macherey-Nagel, Germany) according to the manufacturer's instructions for the isolation of genomic DNA from tissue (user manual). After isolation, genomic DNA was dissolved in 100 μl $ddH_2O$.

### 2.3. Liquid chromatography/tandem mass spectrometry (LC–MS/MS) analysis

Global DNA methylation was detected in at least three different rat liver and kidney samples in each group. Genomic DNA (0.2 μg/μl, dissolved in $ddH_2O$) in a final volume of 50 μl was applied for hydrolysis. After treating with 5 units of nuclease P1 for 1 h at 37 °C, 1 unit of alkaline phosphatase was added and the sample was incubated for 30 min. at 37 °C. The hydrolyzed DNA was transferred onto a cut-off filter (Ultrafree-MC PLCC Centrifugal Filter Unit, 5 kDa, Millipore) and centrifuged for 20 min at 4 °C and 14,000 rpm. The hydrolyzed DNAs were diluted in $ddH_2O$ (1:100) and stored at −70 °C until analysis.

For analysis of 5-methyldeoxycytidine (5-mdC) and 2-deoxyguanosine (2-dG) by LC–MS/MS, 10 μl hydrolyzed DNA solution was injected into the LC-MS/MS system. LC–MS/MS analysis was performed using an Agilent 1100 series LC coupled to an API 3000 triple quadrupole mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems, Darmstadt, Germany). Separation was carried out on a Reprosil Pur ODS 3 150 × 2 mm, 5 μm column (Dr. Maisch GmbH) by gradient elution with 0.1% formic acid (solvent A) and methanol (solvent B) using the following conditions: 90% A and 10% B (starting conditions) followed by an increase to 40% in 3 min and a linear increase to 100% B in 2.5 min, at a flow rate of 0.3 ml/min. Analytes were detected in the positive ion mode at a vaporizer temperature of 400 °C. Data acquisition was performed by multiple reaction monitoring (MRM) of mass transitions m/z 242.17 to 126.0 and m/z 242.17 to 108.95 for 5-mdC and m/z 268.2 to 152.1 for 2-dG. Quantitation of 2-dG and 5-mdC was performed using external standards.

### 2.4. Methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing (BSP)

Bisulfite conversion of genomic DNA was carried out using an Imprint DNA Modification Kit (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's instructions. Genomic DNA (1 μg) from control and high dose animals from each group was used for bisulfite treatment. DNA was eluted in a total volume of 20 μl elution buffer and either used immediately or stored at −80 °C.

MSP analysis of *Cx32*, *e-cadherin*, *p16*, *VHL*, *c-myc*, *Igfbp2*, and *p15* was performed in tissues of rats treated with the highest dose of each non-genotoxic carcinogen. For MSP analyses, primer sequences are listed in Table 1. The MSP products were analyzed by 5% polyacrylamide gel electrophoresis, stained with ethidium bromide and visualized under UV light (GelDoc equipment system, BioRad). To confirm the specificity, DNA was artificially methylated by SssI (CpG) methyltransferase in the presence of s-adenosyl methionine (all from New England Biolabs GmbH, Germany) according to the manufacturer's instructions and used as a positive control for the methylated primer sequence. Negative control reactions were performed by using both primer pairs specific for methylated and unmethylated promotor sequences with DNA not subjected to bisulfite conversion to confirm that unmodified DNA is not amplified in the event of incomplete bisulfite reactions.

Methylation status of the 5′ region of *p16* gene was also determined by bisulfite sequencing (between nt. −478 and 23). After bisulfite modification, PCR was performed using the primers in Table 1. PCR products were purified with High pure PCR product purification kit (Roche Applied Science, Mannheim, Germany), then directly sequenced using an

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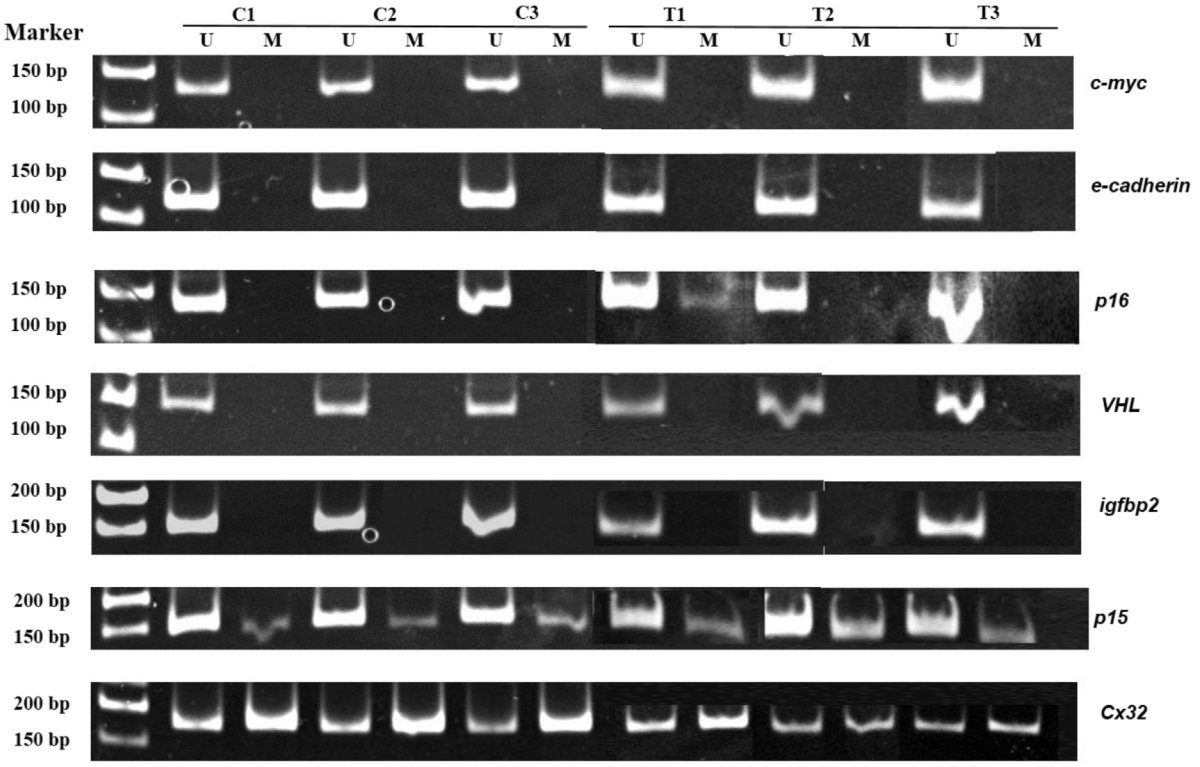



**Fig. 2.** Representative profiles of MSP for the investigated genes using both methylated (M) and unmethylated (U) specific primers in rat liver treated with TCDD (T) at 0.25 μg/kg bw for 28 days and corresponding controls (C).

ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

### 2.5. Methylated DNA immunoprecipitation (MeDIP)

MeDIP-Chip was used to identify alternative genes which have potential methylation alteration. We analyzed tissues of rats treated with the highest dose of each non-genotoxic carcinogen (two control and two treated samples) for MeDIP. Methylated DNA was immunoprecipitated using MagMeDIP kit (mc-magme-048; Diagenode, Liege, Belgium). Briefly, genomic DNA (1 μg was used for immunoprecipitation, IP) was sheared by sonication on ice to generate random fragments of 200–1000 bp and then incubated with anti-5-methylcytosine antibody at 4 °C overnight. After washing four times, the methylated DNA-enriched genomic DNA fraction was eluted with the provided elution buffer.

### 2.6. Real-Time quantitative PCR (qPCR) on immunoprecipitated samples

Before microarray analysis, eluted DNA was used for qPCR at selective regions known to be methylated or unmethylated to monitor the performance of MeDIP enrichment relative to the background. For qPCR, 1/10 and 1/100 of a MeDIP reaction was used. Using a Stratagen Mx3000P qPCR system (Agilent Technologies), each qPCR was performed in a total volume of 25 μL, which contained 12,5 μL DyNAmo 2× SYBRGreen qPCR master mix (Thermo Scientific), 1 μL primer mix (10 μM each primer), 5 μL of immunoprecipitated DNA. Primer sequences of rat *H19*

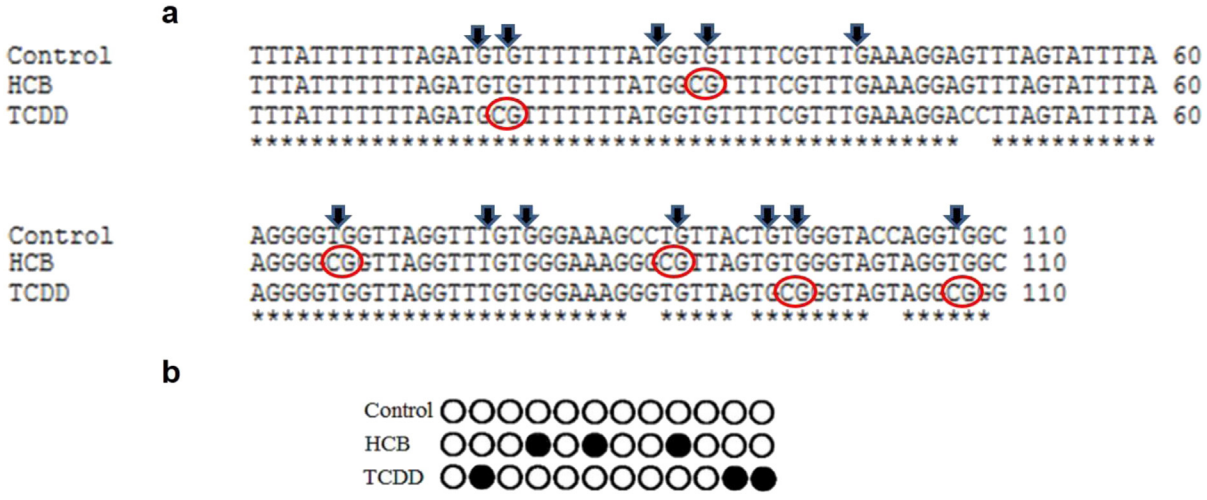



**Fig. 3.** DNA methylation status of p16 the promoter sequence in HCB and TCDD treated rat liver at 200 ppm, bw and 0.25 μg/kg, bw for 28 days, respectively. a) Bisulfite-PCR result of p16 promoter region and b) methylation profiles of CpG dinucleotides of p16 promoter region. Open and closed circles indicate unmethylated and methylated CpG sites, respectively.

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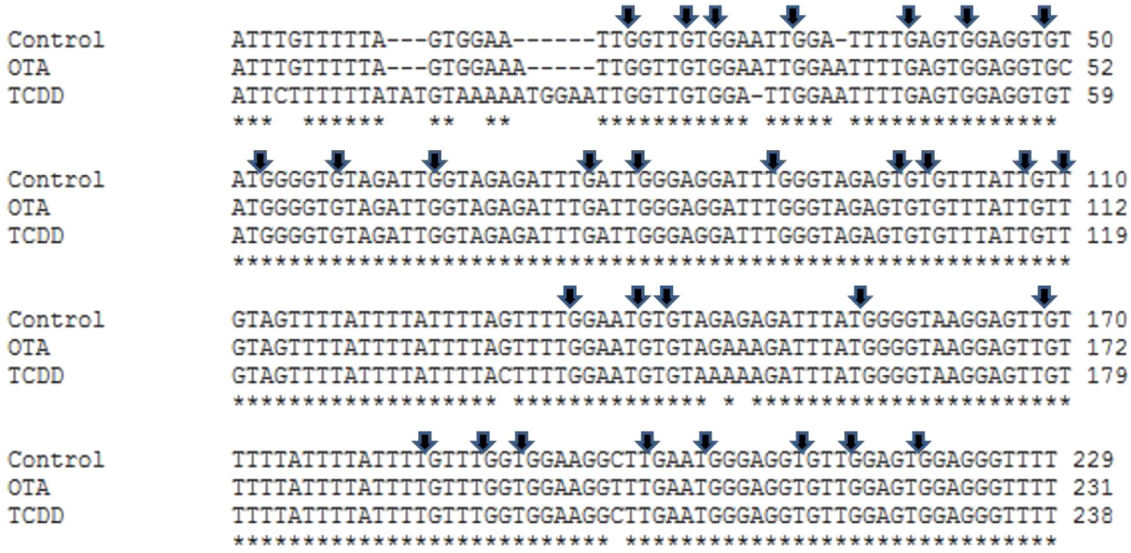



**Fig. 4.** Bisulfite sequencing result of the c-myc promoter region in OTA (210 μg/kg for 90 days) and TCDD (0.25 μg/kg, bw for 28 days) treated rat kidney and liver, respectively.

and *c-myc* were used as methylated and unmethylated controls, respectively (Table 1). The positive and negative controls provided in the Diagenode MagMeDIP kit were also used to confirm the enrichment of methylated DNA by anti-5-methylcytosine antibody. Enrichment was evaluated by calculation of the ratio between input and immunoprecipitated DNA by methylated and unmethylated control sequences.

### 2.7. Microarray analysis and functional data analysis

For microarray analysis, the immunoprecipitated DNA and input DNA were amplified with a whole genome amplification kit (WGA2, Sigma-Aldrich, St. Louis, Missouri, USA). When insufficient amplification product was generated, we used whole genome reamplification kit (WGA3, Sigma-Aldrich, St. Louis, Missouri, USA) to obtain sufficient amounts of immunoprecipitated DNA for microarray analysis. Then, DNA samples were sent to NimbleGen's service laboratory to perform the array experiments using Rat DNA Methylation 3x720K CpG Island Plus RefSeq Promoter Arrays (Cat No: 05924626001, Roche NimbleGen, Reykjavík, Iceland). Data were extracted from scanned images using NimbleScan 2.0 extraction software (Roche NimbleGen, Reykjavík, Iceland). For statistical analysis "comprehensive high-throughput arrays for relative methylation" (CHARM) algorithm was used (Irizarry et al., 2008).

After microarray analysis, functional gene classification and enrichment was performed by DAVID (version 6.7; http://david.abcc.ncifcrf.gov/) (Huang et al., 2009a, 2009b) and PANOGA pathway based analysis tools (Bakir-Gungor and Sezerman, 2011; Bakir-Gungor et al., 2014) to find pathways associated with affected genes and their interacting partners. For DAVID pathway analysis enrichment was performed for the three gene ontology (GO) terms biological process, cellular compartment, and molecular functions and for the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways. PANOGA uses the list methylation affected genes with $p$ values smaller than 0.05 and maps them to a detailed protein–protein interaction (PPI) network. Then, it identifies connected subnetworks that include most of the affected genes from the methylation analysis and the genes they are interacting within the PPI network. It finds all the subnetworks that have at most 50% shared nodes. Then, it finds affected KEGG pathways and assigns a significance value to the degree of being affected by the genes that have altered methylation levels upon being treated with different non-genotoxic carcinogens. Bonferroni correction procedure was applied on the $p$-values of each identified pathway to correct the $p$-values for multiple testing. All the statistically significant KEGG pathways found in an active subnetwork are reported.

## 3. Results

### 3.1. Global DNA methylation

The calibration curves were linear in the concentration range of 0.002–0.2 pmol/μl for 5-mdC and 0.01–10 pmol/μl for 2-dG with a regression factor >0.9915 and >0.9961, respectively. Global DNA methylation in control samples ranged from 4 to 5%. This is consistent with previous studies in which approximately 2–5% of cytosine residues in the mammalian genome were shown to be methylated (Bombail et al., 2004). Compared to vehicle controls, no significant changes in global DNA hypomethylation were observed in the tissues of rats treated with a range of non-genotoxic carcinogens. The effects of non-genotoxic carcinogens on DNA methylation status are summarized in Fig. 1a–c.

### 3.2. Analysis of gene-specific DNA-methylation of selected genes

Representative profiles of MSP for the investigated genes in the liver of rats treated with TCDD and corresponding controls are shown in Fig. 2. CpG promoter DNA methylation was not detected in *c-myc*, *e-cadherin* and *VHL* promoter regions in vehicle controls and no alterations were seen in response to treatment with any of the non-genotoxic kidney and liver carcinogens. MSP analysis showed *p16* promoter methylation in the livers of 1 of 3 rats treated with TCDD and 1 out of 3 rats treated with HCB. Bisulfite sequencing results further confirmed the low methylation status of *p16* promoter in TCDD and HCB treated rat liver (Fig. 3).

In contrast to *c-myc*, *e-cadherin*, *VHL* and *p16*, both *Cx32* and *p15* promoter regions were found to be partially methylated in controls, indicating that CpG island methylation is present in normal male rat liver and kidney tissue. *Igfbp2* promoter methylation was detected in 2/3 kidneys of rats treated with d-limonene, chloroform and DCB. However, the same methylation pattern and incidence was also observed in the corresponding controls, indicating that *Igfbp2* promoter methylation was not treatment related in the kidney. Also, DNA methylation was not detected in *Igfbp2* promoter regions in vehicle controls and rat liver treated with TCDD, HCB and MPY.

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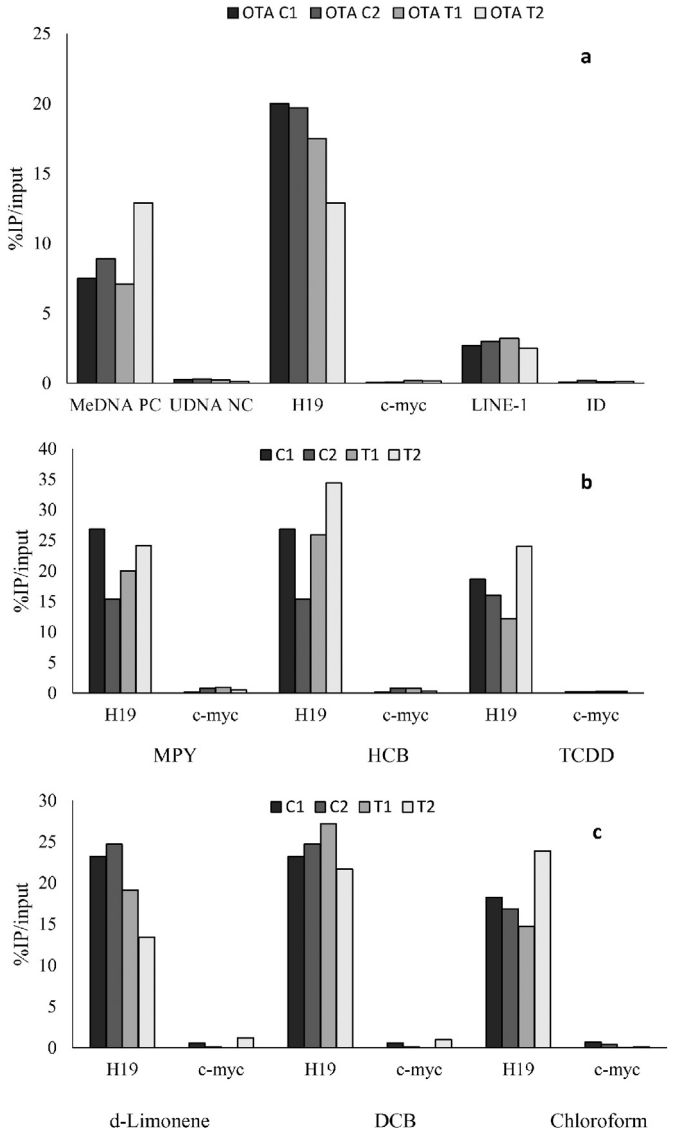



**Fig. 5.** q-PCR validation of MeDIP results. MeDNA PC and UDNA NC: internal positive and negative controls provided in the Diagenode MeDIP kit. LINE-1, H19, ID and cmyc, rat genes used to confirm the immunoprecipitation result. C1, C2: control samples; T1, T2: non-genotoxic carcinogen treated samples: a) kidneys of male rats treated with OTA at 210 μg/kg bw for 90 days, b) livers of female rats treated with HCB (200 ppm in diet), MPY (1000 ppm in diet) and TCDD (0.25 μg/kg bw) for 28 days, c) kidneys of male rats treated with d-limonene (150 mg/kg bw), DCB (300 mg/kg bw) and chloroform (300 mg/kg bw) for 28 days.

### 3.3. Analysis of genome-wide DNA-methylation

Rat DNA methylation 3x720K CpG Island Plus RefSeq Promoter Arrays were used to detect 15,600 transcript, 15,287 promoter region and 15,790 CpG sites. After immunoprecipitation, we examined the enrichment of four single-copy genes as well as the positive and negative controls provided in the Diagenode MagMeDIP kit to confirm the enrichment of methylated DNA by anti-5-methylcytosine antibody. The hypomethylation status of c-*myc* gene promoter region was independently shown by bisulfite sequencing (Fig. 4), whereas the 5′-upstream region of the *H19* gene has been found to be highly methylated in the adult rat by bisulfite sequencing (Manoharan et al., 2004). Thus, *c-myc* and *H19* could be employed as negative and positive controls, respectively (Chen et al., 2010). Repetitive sequences such as long/short interspersed nucleotide elements (*LINE* and *SINE*) in retrotransposons are methylated at different levels. Thus, *LINE-1* methylation status has been shown to be a good indicator of global genomic DNA methylation (Asada et al., 2006; Florea, 2013; Yang et al., 2004). Therefore, we also investigated the enrichment rate of *LINE-1* and *ID* (Identifier) elements which are members of a family of *SINEs* to confirm global DNA methylation level. In our results, only the *H19* gene and the positive internal control were enriched in immunoprecipitated DNA (Fig. 5a, b, c). *LINE-1* showed little enrichment in control and OTA treated kidney samples (Fig. 5a) which is consistent with the previous reports that *LINE-1* was hypomethylated in rat liver (Asada et al., 2006; Chen et al., 2010).

### 3.4. Functional data analysis

Although we detected a number of methylation changes, most of the changes were less than 10% change in the methylation level and thus not considered to be biologically relevant. We therefore, focused our functional data analysis on differentially methylated genes that showed more than 10% difference in methylation level. Using these criteria, only MPY, TCDD and OTA produced some changes in the methylation level in their respective target organ, whereas the remaining non-genotoxic carcinogens did not induce significant changes under the conditions of our study. MPY and TCDD altered the methylation status of a few genes listed in Table 2. OTA treatment for 90 days resulted in a significant number of methylation changes (Table 3), including hypermethylation of genes such as *cullin 2* (*Cul2*) and *discs large homolog 2* (*Dlg2*) and hypomethylation of *3-phosphoinositide dependent protein kinase-1* (*Pdpk1*), *copine IV* (*Cpne4*) and *regulatory associated protein of mTOR* (*Rptor*) genes.

The web-based annotation tool DAVID (Huang et al., 2009a, 2009b) revealed that only few GO category biological process regulation of phosphate metabolic process (*Pdpk1*, *Dgkg*, *Rptor*), positive regulation of catalytic activity and molecular function (*Pdpk1*, *Dgkg*, *Htr4*), activation of protein kinase activity (*Pdpk1*, *Dgkg*) and regulation of protein localization (*Pdpk1*, *Tbc1d1*) were enriched whereas the KEGG pathway mTOR signaling pathway (*Pdpk1*, *Rptor*) was found to be significantly enriched in the gene group exhibiting hypomethylation byOTA (Table 4). Furthermore, pathway mapping of hypomethylated genes showed that the GO category molecular function terms vitamin binding and protein kinase binding were significantly enriched (Table 4). The molecular function GO terms showed that hypermethylated genes corresponded to protein heterodimerization/dimerization activity (Table 4). In addition, PANOGA analysis revealed a link to several signaling pathways such as neurotrophin, Fc epsilon RI, T cell receptor, adipocytokine and *ErbB* with most affected genes of *Dock1* and *Pdpk1* in response to OTA.

For MPY, functional classification was not possible due to the limited number of genes affected. However, PANOGA analysis revealed that differentially methylated genes in livers of MPY treated rats are linked to several cancers such as leukemia, renal cancer, thyroid cancer and bladder cancer, and identified an association with aldosterone-regulated sodium reabsorption, homologous recombination and notch signaling KEGG pathways. For TCDD, the molecular function GO terms showed that differentially methylated genes corresponded to protein homodimerization/dimerization activity and identical protein binding (Table 4). The cellular component GO terms revealed that the target genes were related to cell surface, while no relation were observed in biological process GO terms (Table 4). PANOGA identified an association with adherens junctions and the pentose phosphate pathway, and thyroid and colorectal cancers in response to TCDD.

## 4. Discussion

Altered DNA methylation has been suggested to play a key role as an epigenetic mechanism in chemical-induced carcinogenesis (Watson and Goodman, 2002; Moggs et al., 2004). In the present study, we aimed to investigate the effects of a range of non-genotoxic carcinogens (TCDD, HCB, MPY, DCB, d-limonene, chloroform and OTA) on global, gene-specific CpG island and genome-wide DNA methylation to

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determine if altered DNA methylation may provide a source of new biomarkers for early detection of non-genotoxic carcinogenesis. We applied a well characterized in vivo 4-week protocol that has been previously used to investigate early mechanisms associated with TCDD, HCB, MPY, DCB, d-limonene and chloroform carcinogenicity (Mally and Chipman, 2002). Under these conditions, target tissue specific alteration of gap junction plaques containing Cx32 and increased renal cell proliferation (in response to rat kidney carcinogens) were commonly observed in the absence of major histopathological changes. For OTA, which also led to a significant increase in renal cell proliferation and associated changes in gene expression, a later timepoint (90 days) was also included (Rached et al., 2007; Adler et al., 2009).

In this study, we found no significant changes in global DNA methylation in target tissues of rats treated with a range of non-genotoxic liver and kidney carcinogens as compared to vehicle controls. While these findings suggest that alterations in global DNA methylation are not an early event associated with doses of studied non-genotoxic carcinogens known to induce tumors after chronic exposures, they contrast reports by others showing early hypomethylation in response to chemicals such as rutin, bile acids, dichloroacetic acid, trichloroacetic acid and phenobarbital (Bachman et al., 2006; Pereira et al., 2004; Tao et al., 2005).

Our data suggest that global DNA hypomethylation, which is frequently observed in tumors, may require sustained exposure. However, determination of 5-mC levels may not be sufficiently sensitive to detect epigenetic changes due to gene-specific methylation and subsequent silencing of tumor suppressor genes. Since it is well established that many non-genotoxic carcinogens influence the expression of genes involved in cell cycle and apoptosis (e.g. *c-myc*, *p15*, *p16*), we selected a number of such genes that might be modulated by DNA methylation. In our study, CpG island methylation was not detected in the promoter regions of *c-myc*, *e-cadherin*, *VHL*, *p15* and *igfbp2* genes using MSP after treatment of rats with the selected non-genotoxic liver and kidney carcinogens. No changes were also observed in CpG island methylation status of *Cx32* gene in response to non-genotoxic carcinogens, which suggests that loss of *Cx32* containing plaques which we previously identified as a common, early event in non-genotoxic carcinogenesis (Mally and Chipman, 2002) may occur by mechanisms that do not involve changes in DNA methylation.

Since this targeted profiling approach revealed no significant methylation changes, we applied MeDIP combined with microarrays, which

**Table 2**
Differentially methylated genes identified in livers of rats treated with MPY (1000 ppm, bw for 28 days) or TCDD (0.25 μg/kg, bw for 28 days). Only genes that showed more than 10% differential methylation are listed.

| Gene symbol | Gene ID | Gene name | Description[a] |
|---|---|---|---|
| **MPY** | | | |
| ***Hypermethylation*** | | | |
| Snrnp40 | 313056 | Small nuclear ribonucleoprotein 40 kDa (U5) | Spliceosome pathway |
| Brd8 | 291691 | Bromodomain containing 8 | Positive regulation of transcription from RNA polymerase II promoter, histone H2A acetylation, histone H4 acetylation, INO80 family mediated chromatin remodeling pathway |
| Arhgap44 | 303222 | Rho GTPase activating protein 44 | Exocytosis, positive regulation of GTPase activity, signal transduction |
| Rexo1 | 314630 | REX1, RNA exonuclease 1 homolog | Nucleic acid phosphodiester bond hydrolysis, ribosome biogenesis pathway |
| RGD1311575 | | Uncharacterized protein KIAA1211 | |
| RGD1560028 | 500157 | Similar to RIKEN cDNA C130060K24 gene | Neuropeptide signaling pathway |
| RGD1563065 | 289633 | NACHT and WD repeat domain containing 2 | |
| Nwd2 | | | |
| Zscan5b | 292566 | Zinc finger and SCAN domain containing 5B | Regulation of transcription |
| Pcsk2 | 25121 | Proprotein convertase subtilisin/kexin type 2 | Islet amyloid polypeptide processing, peptide hormone processing, proteolysis, melanocortin system pathway |
| Itk | 363577 | IL2-inducible T-cell kinase | Activation of phospholipase C activity, adaptive immune response, cytokine production, phosphatidylinositol 3-kinase class I signaling pathway, chemokine mediated signaling pathway, T cell receptor signaling pathway |
| Cpne4 | 367160 | Copine IV | |
| ***Hypomethylation*** | | | |
| Eif4g3 | 298573 | Eukaryotic translation initiation factor 4 gamma, 3 | Positive regulation of meiosis I, positive regulation of protein phosphorylation, positive regulation of translation, myocarditis pathway |
| Plce1 | 114633 | Phospholipase C, epsilon 1 | G-protein coupled receptor signaling pathway, Ras protein signal transduction, activation of MAPK activity, calcium/calcium-mediated signaling pathway, inositol phosphate metabolic pathway, phosphatidylinositol 3-kinase signaling pathway |
| **TCDD** | | | |
| ***Hypermethylation*** | | | |
| Kcnk13 | 64120 | Potassium channel, subfamily K, member 13 | Potassium ion transmembrane transport, regulation of ion transmembrane transport |
| Abcg1 | 85264 | ATP-binding cassette, subfamily G (WHITE), member 1 | Amyloid precursor protein catabolic process, cholesterol efflux, cholesterol homeostasis |
| Ubash3b | 315579 | Ubiquitin associated and SH3 domain containing, B | Negative regulation of platelet aggregation, negative regulation of protein kinase activity, peptidyl-tyrosine dephosphorylation |
| Ly86 | 291359 | Lymphocyte antigen 86 | Positive regulation of lipopolysaccharide-mediated signaling pathway |
| Dscaml1 | 315615 | Down syndrome cell adhesion molecule-like 1 | Brain development, dendrite self-avoidance, embryonic skeletal system morphogenesis |
| Fbxl16 | 494223 | F-box and leucine-rich repeat protein 16 | |
| ***Hypomethylation*** | | | |
| Cpne4 | 367160 | Copine IV | |

[a] Regarding to "Rat genome database (RGD)" (Shimoyama et al., 2015).

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**Table 3**
Differentially methylated genes identified in kidneys of rats treated with OTA (210 μg/kg, bw for 90 days). Only the genes that showed more than 10% differential methylation are listed.

| OTA | | | |
|---|---|---|---|
| **Gene symbol** | **Gene ID** | **Gene name** | **Description**[a] |
| ***Hypermethylation*** | | | |
| Copg2 | 301742 | Coatomer protein complex, subunit gamma 2 | Intra-golgi vesicle-mediated transport |
| Tbc1d5 | 501088 | TBC1 domain family, member 5 | Positive regulation of autophagy, positive regulation of receptor internalization, response to starvation |
| Ano6 | 315272 | Anoctamin 6 | Positive regulation of potassium ion export, activation of blood coagulation via clotting cascade, blood coagulation |
| Rsrc1 | 361956 | Arginine/serine-rich coiled-coil 1 | Alternative mRNA splicing, via spliceosome (ortholog); mRNA splicing, via spliceosome (ortholog); nucleocytoplasmic transport (ortholog). |
| Arap2 | 305367 | ArfGAP with RhoGAP domain, Ankyrin repeat and PH domain 2 | Positive regulation of GTPase activity, regulation of ARF GTPase activity, signal transduction |
| Micu3 | 364601 | Mitochondrial calcium uptake family, member 3 | |
| Zfp213 | 287094 | Zinc finger protein 213 | Regulation of transcription |
| Olr233 | 293377 | Olfactory receptor 233 | Detection of chemical stimulus involved in sensory perception of smell, G-protein coupled receptor signaling pathway |
| Dlg2 | 64053 | Disks, large homolog 2 (*Drosophila*) | Neuronal ion channel clustering, receptor clustering, negative regulation of phosphatase activity |
| Dock1 | 309081 | Dedicator of cyto-kinesis 1 | Cell migration, hematopoietic progenitor cell differentiation, positive regulation of GTPase activity |
| Tcf3 | 171046 | Transcription factor 3 | Positive regulation of gene expression, positive regulation of transcription, B cell lineage commitment, acute myeloid leukemia pathway; arrhythmogenic right ventricular cardiomyopathy pathway, basal cell carcinoma pathway |
| Maoa | 29253 | Monoamine oxidase A | Phenylethylamine metabolic process, serotonin metabolic process, dopamine catabolic process, arginine and proline metabolic pathway, glycine, serine and threonine metabolic pathway, histidine metabolic pathway |
| Kcnd2 | 65180 | Potassium voltage-gated channel, Shal-related subfamily, member 2 | Action potential, cellular response to drug, cellular response to mechanical stimulus, excitatory synaptic transmission pathway, long term potentiation |
| Vom1r6 | 494231 | Vomeronasal 1 receptor 6 | G-protein coupled receptor signaling pathway |
| Olr1014 | 404920 | Olfactory receptor 1014 | Detection of chemical stimulus involved in sensory perception of smell G-protein coupled receptor signaling pathway |
| Cul2 | 361258 | Cullin 2 | Protein catabolic process, hypoxia inducible factor pathway, neddylation pathway, proteasome degradation pathway involving cullin-dependent ubiquitin ligases |
| ***Hypomethylation*** | | | |
| Nrde2 | 314381 | NRDE-2, necessary for RNA interference, domain containing | |
| Apitd1 | 100534597 | Apoptosis-inducing, TAF9-like domain 1 | Cellular response to DNA damage stimulus, DNA repair, replication fork processing |
| Htr4 | 25324 | 5-Hydroxytryptamine (serotonin) receptor 4, G protein-coupled | Adenylate cyclase-activating G-protein coupled receptor signaling pathway, gamma-aminobutyric acid signaling pathway, serotonin receptor signaling pathway, calcium/calcium-mediated signaling pathway |
| Tbc1d1 | 360937 | TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1 | Regulation of protein localization |
| Dgkg | 25666 | Diacylglycerol kinase, gamma | Neuron development, Glycerolipid metabolic pathway, glycerophospholipid metabolic pathway, phosphatidylinositol 3-kinase signaling pathway |
| Slc16a14 | 316578 | Solute carrier family 16, member 14 | |
| Cpne4 | 367160 | Copine IV | |
| Pdpk1 | 81745 | 3-Phosphoinositide dependent protein kinase-1 | Focal adhesion assembly, activation of protein kinase B activity, calcium-mediated signaling, angiotensin II signaling pathway, FasL mediated signaling pathway, fibroblast growth factor signaling pathway |
| Spon1 | 64456 | Spondin 1, extracellular matrix protein | Cell adhesion |
| Efcab5 | 363653 | EF-hand calcium binding domain 5 | |
| Ccdc113 | 291847 | Coiled-coil domain containing 113 | Cilium assembly |
| Col6a5 | 501047 | Collagen, type VI, alpha 5 | |
| Dock3 | 315992 | Dedicator of cyto-kinesis 3 | Positive regulation of GTPase activity, small GTPase mediated signal transduction |
| Spop | 287643 | Speckle-type POZ protein | Glucose homeostasis, negative regulation of sequence-specific DNA binding transcription factor activity, negative regulation of transcription from RNA polymerase II promoter, Hedgehog signaling pathway |
| Npc1l1 | 432367 | NPC1-like 1 | Response to drug, cholesterol biosynthetic process, cholesterol homeostasis |
| Ogdh | 360975 | Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide) | 2-Oxoglutarate metabolic process, NADH metabolic process, succinyl-CoA metabolic process, citric acid cycle pathway, lysine degradation pathway, tryptophan metabolic pathway |
| Oat | 64313 | Ornithine aminotransferase | Protein hexamerization, arginine and proline metabolic pathway |
| RGD1560242 | 499334 | Similar to RIKEN cDNA 1700028P14 | |
| Mefv | 58923 | Mediterranean fever | Response to lipopolysaccharide, response to silicon dioxide, inflammatory response, NOD-like receptor signaling pathway |
| Rptor | 287871 | Regulatory associated protein of MTOR, complex 1 | Positive regulation of endothelial cell proliferation, regulation of phosphorylation, cell growth, mTOR signaling pathway, insulin signaling pathway |

[a] Regarding to "Rat genome database (RGD)" (Shimoyama et al., 2015).

determines the distribution of DNA-methylation within promoters or in the entire genome, to identify genes modulated by treatment with non-genotoxic carcinogens (Chen et al., 2010, 2012). We found methylation changes using the method of MeDIP and microarray assay for MPY, TCDD and OTA while HCB, DCB, d-limonene and chloroform did not produce significant methylation changes.

In the livers of rats treated with TCDD, our data showed that few genes such as *Kcnk13*, *Abcg1*, *Ubash3b*, *Ly86*, *Fbxl16* and *Serhl2* were hypermethylated whereas only Cpne4 was hypomethylated. Due to the limited number of differentially methylated genes pathway mapping using DAVID revealed no association with biological process GO term in response to TCDD.

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**Table 4**
GO categories of the differentially methylated genes for OTA and TCDD by DAVID annotation tool.

| Category | Term | | Gene count[a] | P value[*] | Genes | Benjamini |
|---|---|---|---|---|---|---|
| **OTA** | | | | | | |
| ***Hypomethylation*** | | | | | | |
| GOTERM_BP_FAT | GO:0042325 | Regulation of phosphorylation | 3 | 0.034 | Pdpk1, Dgkg, Rptor | 0.994 |
| GOTERM_BP_FAT | GO:0019220 | Regulation of phosphate metabolic process | 3 | 0.036 | Pdpk1, Dgkg, Rptor | 0.935 |
| GOTERM_BP_FAT | GO:0043085 | Positive regulation of catalytic activity | 3 | 0.041 | Pdpk1, Dgkg, Htr4 | 0.874 |
| GOTERM_BP_FAT | GO:0044093 | Positive regulation of molecular function | 3 | 0.052 | Pdpk1, Dgkg, Htr4 | 0.863 |
| GOTERM_BP_FAT | GO:0032147 | Activation of protein kinase activity | 2 | 0.071 | Pdpk1, Dgkg | 0.890 |
| GOTERM_BP_FAT | GO:0032880 | Regulation of protein localization | 2 | 0.092 | Pdpk1, Tbc1d1 | 0.909 |
| GOTE R M_MF_FAT | GO:0019842 | Vitamin binding | 2 | 0.074 | Ogdh, Oat | 0.977 |
| GOTE R M_MF_FAT | GO:0019901 | Protein kinase binding | 2 | 0.088 | Pdpk1, Rptor | 0.896 |
| KEGG_PATHWAY | rno04150 | mTOR signaling pathway | 2 | 0.065 | Pdpk1, Rptor | 0.719 |
| ***Hypermethylation*** | | | | | | |
| GOTE R M_MF_FAT | GO:0046982 | Protein heterodimerization activity | 3 | 0.021 | Dlg2, Kcnd2, Tcf3 | 0.767 |
| GOTE R M_MF_FAT | GO:0046983 | Protein dimerization activity | 3 | 0.096 | Dlg2, Kcnd2, Tcf3 | 0.969 |
| GOTE R M_CC_FAT | GO:0014069 | Postsynaptic density | 2 | 0.067 | Dlg2, Kcnd2 | 0.997 |
| **TCDD** | | | | | | |
| ***Hypermethylation*** | | | | | | |
| GOTE R M_MF_FAT | GO:0042803 | Protein homodimerization activity | 2 | 0.052 | Dscaml1, Abcg1 | 0.912 |
| GOTE R M_MF_FAT | GO:0046983 | Protein dimerization activity identical | 2 | 0.086 | Dscaml1, Abcg1 | 0.869 |
| GOTE R M_MF_FAT | GO:0042802 | Protein binding | 2 | 0.096 | Dscaml1, Abcg1 | 0.78 |
| GOTE R M_CC_FAT | GO:0009986 | Cell surface | 2 | 0.062 | Dscaml1, Abcg1 | 0.4 |

[a] Indicates number of genes in the dataset that belong to this category.
[*] P-values were calculated with a modified Fisher exact test.

We observed that a few genes such as *Snrnp40*, *Brd8*, *Zscan5b*, *Pcsk2*, *Itk* and *Cpne4* were hypermethylated while *Eif4g3* and *Plce1* were hypomethylated in response to MPY. We could not identify an association between differentially methylated genes and signaling pathways or an apparent link to MPY mediated gene expression changes (Uehara et al., 2008b).

As shown in Table 3, 90-day OTA treatment revealed more differentially methylated genes including hypermethylation of *Tbc1d5*, *Ano6*, *Arap2*, *Dlg2*, *Cul2* and hypomethylation of *Cpne4*, *Pdpk1*, *Dock3*, *Spop*, *Ogdh*, *Oat* and *Rptor*. Pathway mapping of these genes indicated phosphate metabolic process and protein kinase activity as biological process GO term, and mTOR signaling pathway enriched as KEGG category. *mTOR* is a *serine–threonine kinase* that plays a critical role in the positive regulation of cell growth, survival and other cellular functions primarily through direct interaction with *Raptor* (Guertin and Sabatini, 2007). In a previous study, short-term OTA treatment (210 μg/kg body weight for 1, 3, 7, and 14 days) of *tuberous sclerosis 2* (*Tsc2*) mutant Eker rats resulted in activated mTOR signaling (Stemmer et al., 2007). It has been suggested that OTA has mitogenic properties by down-regulation of *IGFBP-4*, a negative regulator of the (IGF)-PI3K-AKT mitogenic pathway in Eker and wild-type rats, but only Eker rats showed a significant increase of preneoplastic lesions (Stemmer et al., 2007). Deregulated genes involved in the PI3K-AKT-Tsc2-mammalian target of rapamycin (mTOR) signaling suggested involvement of *Tsc2* in OTA-mediated toxicity and carcinogenicity (Horvath et al., 2002; Gekle et al., 2005; Stemmer et al., 2007). Additionally, OTA was found to increase the phosphorylation of *protein kinase C* and downstream activation of the *MAPK-ERK* system in kidney samples of male rats fed a carcinogenic dose of OTA (Marin-Kuan et al., 2007). The ErbB receptors signal through Akt, *MAPK* and other pathways regulate cell proliferation, migration, differentiation, apoptosis and cell motility which could be relevant to OTA nephrocarcinogenicity.

Interestingly, *copine 4* (*Cpne4*) gene, a member of the calcium-dependent, phospholipid-binding protein family which is involved in membrane trafficking, mitogenesis and development, was differentially methylated in response to MPY, TCDD and OTA. Copine genes encode proteins which contains two type II C2 domains in the amino-terminus and an A domain-like sequence in the carboxy-terminus. While copines are calcium-dependent phospholipid-binding proteins with intrinsic kinase activity (Tomsig and Creutz, 2000), the A domain mediates interactions with integrins and extracellular ligands. Copine target proteins are involved in intracellular signaling pathways, such as MEK1, MEK1 [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase], protein phosphatase 5, and the CDC42-regulated kinase, ubiquitin (or NEDD8)-conjugating enzymes and transcription modulators (Tomsig et al., 2003, 2004). Since *Cpne4* was differentially methylated in response to MPY, TCDD and OTA, it may have the potential to serve as an early biomarker of non-genotoxic carcinogenesis.

In conclusion, our analyses did not identify genome-wide methylation changes in response to short-term treatment with HCB, d-limonene, DCB and chloroform. While TCDD and MPY showed methylation changes in a few genes, this did not reveal a significantly affected pathway. However, 90-day treatment with OTA induced methylation changes in genes involved in phosphate metabolic process, protein kinase activity and mTOR cell signaling which could contribute to OTA carcinogenicity. Overall, our data suggest that altered DNA methylation may in some cases, but not all, be an early event associated with non-genotoxic carcinogens, but highlight *Cpne4* as a potential biomarker for nongenotoxic carcinogenesis.

## Transparency document

The Transparency document associated with this article can be found, in online version.

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