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Genome-wide study of DNA methylation alterations in response to Diazinon exposure *in vitro*

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Conflict of Interests

The authors declare that they have no conflicts of interest.

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Abstract

Pesticide exposure has repeatedly been associated with cancers. However, molecular mechanisms are largely undetermined. In this study, we examined whether exposure to diazinon, a common organophosphate that has been associated with cancers, could induce DNA methylation alterations. We conducted genome-wide DNA methylation analyses on DNA samples obtained from human hematopoietic K562 cell exposed to diazinon and ethanol using the Illumina Infinium HumanMethylation27 BeadChip. Bayesian-adjusted t-tests were used to identify differentially methylated gene promoter CpG sites. We identified 1069 CpG sites in 984 genes with significant methylation changes in diazinon-treated cells. Gene ontology analysis demonstrated that some genes are tumor suppressor genes, such as TP53INP1 (3.0-fold, q-value<0.001) and PTEN (2.6-fold, q-value<0.001), some genes are in cancer-related pathways, such as HDAC3 (2.2-fold, q-value=0.002), and some remain functionally unknown. Our results provided direct experimental evidence that diazinon may modify gene promoter DNA methylation levels, which may play a pathological role in cancer development.

Keywords

Diazinon exposure; DNA methylation alteration; carcinogenesis

1. Introduction

Pesticides are widely used and pervasive in our environment (Weichenthal et al., 2010). Our dependence upon pesticides is increasing. Diazinon, a common organophosphate (OP) insecticide, is registered for a variety of uses on plants and animals. In 2004, approximately 4 million pounds of diazinon were applied in agricultural settings in the US (Watterson, 1999). Although diazinon has been classified as “not likely a human carcinogen” by Environmental Protection Agency (EPA) based on genotoxicity and mutagenicity tests (Bianchi et al., 1994; EPH, 1997), *in vitro* and animal studies have suggested that diazinon induced carcinogenicity (Galloway and Handy, 2003; Handy et al., 2002; Hatjian et al., 2000; Matsuoka et al., 1979; Tisch et al., 2002). In addition, diazinon has been repeatedly associated with various cancers in human studies, including leukemia (Beane Freeman et al., 2005), non-Hodgkin’s lymphoma (NHL) (Cantor et al., 1992; Waddell et al., 2001; Zahm et al., 1993), lung (Alavanja et al., 2004; Beane Freeman et al., 2005; Pesatori et al., 1994), brain (Davis et al., 1993) and prostate cancers (Band et al., 2011). The elevated cancer risk following exposure to diazinon indicates a gap in the current knowledge of pesticide carcinogenicity, and provides evidence that diazinon and other pesticides may cause cancer through alternative mechanisms, such as epigenetic changes (Alavanja, 2009; Skinner and Anway, 2007).

Methylation of 5’-CpG islands in gene promoter regions has consistently been found in malignant tissues and is indicative of a critical early change at the molecular level in the development of human cancers (Issa, 2004). DNA methylation alterations in gene promoters have also been repeatedly found in relation to exposure to various environmental chemicals, including several pesticides (Baccarelli and Bollati, 2009). Animal studies have shown that exposure to pesticides, such as vinclozolin, methoxychlor, and dichlorvos, induced promoter DNA methylation alterations of multiple genes, including lysophospholipase, G protein-

coupled receptor 33 (GPR33), potassium voltage-gated channel, Isk-related family, member 2 (KCNE2), annexin A1 (ANXA1), etc. (Anway and Skinner, 2006; Guerrero-Bosagna et al., 2010; Hathaway et al., 1991). Genomic DNA methylation content, also referred to as the global methylation level, has been found in blood leukocyte DNA to be inversely associated with the plasma levels of pesticide residues in an Arctic population (Rusiecki et al., 2008), and a similar observation was made in a Korean population (Kim et al., 2010). However, previous studies have been limited to the evaluation of very small sets of candidate methylation markers. The scientific evidence concerning the effect of pesticide exposure, such as diazinon, on DNA methylation alteration is still limited, and DNA methylation alterations are not considered in carcinogenicity testing by the EPA or other agencies. To the best of our knowledge, no genome-scale investigation has been conducted to identify epigenomic loci that are sensitive to diazinon exposure. Study of epigenomics in relation to pesticide exposure will provide information on whether pesticides have epigenetic effects, which play important roles in cancer etiology, in addition to those traditional cytotoxicity and standard genotoxicity assessments would predict. The purpose of the present study was to conduct a genome-wide investigation to examine comprehensively whether exposure to diazinon, a commonly used OP that has been associated with several cancers in human studies, could induce DNA methylation alterations *in vitro*.

2. Materials and Methods

2.1. Exposure of human K562 cells to diazinon

Recently, *in vitro* system has been used to determine if chemical exposure alters DNA methylation (Bachman et al., 2006; Watson et al., 2004). The human K562 cell line was derived from erythroblastic leukemia and can differentiate into recognizable progenitors of the erythrocytic, granulocytic and monocytic series (Andersson et al., 1979; Baker et al., 2001; Lozzio et al., 1981). Studies using K562 cells have demonstrated that DNA methylation can be altered by treatment with agents such as interleukin-6 and cadmium (Hodge et al., 2001; Huang et al., 2008), and in this study, K562 cells were exposed to diazinon. The K562 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in RPMI-1640 supplemented with 10% fetal calf serum, 100 µg/ml penicillin, and 100 U/ml streptomycin (Invitrogen, Carlsbad, CA). Diazinon was purchased from ChemService (West Chester, PA) and stock solutions were dissolved in ethanol. K562 cells were exposed to diazinon at doses of 0.001, 0.01, and 0.1 µM, or ethanol (control) for different time periods (12, 24, 48 and 72 hours). All the experiments were conducted in triplicates. Viability using triplicate samples was first assessed by the trypan blue exclusion assay using a hemocytometer for cell counting under an inverted microscope. Additionally, cell cytotoxicity was determined using the luciferase-based ToxiLight® (Lonza, Rockland, ME) assay system. Luminescence produced by luciferase is proportional to adenylate kinase release in the ToxiLight® assay and was measured in relative light units (RLUs) using a Fusion™ Universal Microplate Analyzer (Packard BioScience Company, Meriden, CT). These exposure dosages did not significantly affect cell viability (Supplemental Table 1) or show cytotoxic effects (Supplemental Figure 1), and are similar to exposure levels experienced by pesticide applicators in real life (Arcury et al., 2007; Arcury et al., 2009; Barr et al., 2005; Fenske et al., 2002). DNA was prepared with a Wizard Genomic DNA purification kit (Promega Corp, Madison, WI), quantitated, and diluted into aliquots of 25ng/µl of DNA for the genome-wide DNA methylation analysis.

2.2. Genome-wide examination of DNA methylation alterations

Genome-wide DNA methylation examination was performed on DNA samples obtained from biological triplicate cells exposed to diazinon at a dose of 0.1µM for 12 hours using

Illumina Infinium Human Methylation27 BeadChip, which contains 27,578 individual CpG sites covering 14,000 genes. The selection of the dose and time of exposure used in the current genome-wide investigation was based on our previous gene-specific methylation pilot experiment, in which K562 cells were exposed to several pesticides at different doses over different periods of time. Cells treated with pesticides at doses 0.1 μ M for 12 hours or more demonstrated DNA methylation changes, and increasing doses did not generate significant differences in DNA methylation levels (unpublished data). Therefore, for genome-wide DNA methylation analysis, samples exposed at a dose of 0.1 μ M for 12 hours were used. This Illumina whole genome microarray uses probes of 50-mer oligonucleotides covalently coupled to microspheres, and provides a quantitative methylation measurement at the single-CpG-site level (Baker, 2010; Chen et al., 2011). 500 ng of DNA was used to perform bisulfite conversion using the EZ-96 DNA Methylation Kit (Zymo Research, Orange, CA) following Illumina's protocol. Samples and controls for this study were dispersed in a 96-well plate with other samples to avoid inter-chip effects. Illumina BeadChips were scanned with an iScan and then analyzed by the GenomeStudio software. All experiments were conducted at the Genomics Core Facility of Northwestern University in accordance with the manufacturer's protocols. The 600 negative control probes and bisulfite conversion probes provided by Human Methylation27 were used to identify weak bead types and failed samples. As suggested by the manufacturer, samples with <75% detected loci were re-run. As per Northwestern University Genomic Core Facility procedures, 5% replicates of 96 samples were interspersed with study samples in a 96-well plate using pseudo-participant IDs to mask their origin from laboratory personnel. The concordance rates of the duplicate samples were 98%. To further quantify and remove any inter-array effects, we developed our own quality control (QC) procedure by including commercially available known unmethylated (normal B-lymphocytes (Coriell: NA10923), Camden, NJ) and methylated (colon cancer cells (ATCC: HTB-38), Manassas, VA) control samples in each run (Du et al., 2010). Unmethylated and methylated samples were mixed in ratios of 10:0, 9:1, 7.5:2.5, 5:5, and 0:10. For ten genes known to be hyper-methylated in cancer tissues (Bibikova et al., 2006), the resulting percentage of methylated cytosines over the sum of methylated and unmethylated cytosines (%mc) was plotted against the mixture ratio (Supplemental Figure 2). As illustrated, %mc varied directly with the mixture ratio on duplicate runs (mean $r=0.99$).

2.3. Bioinformatics/biostatistics analysis

The Illumina Infinium methylation microarray data was processed by the Bioconductor lumi package (Du et al., 2008). The GenomeStudio output data first went through a quality assurance (QA)/QC step. For the samples passing the QA/QC step, we performed a color balance adjustment of methylated and unmethylated probe intensities between two color channels using the smooth quantile normalization method. The methylated and unmethylated probe intensities were then normalized using the Simple Scaling Normalization (SSN) method. Methylation M-value (log₂ ratio of methylated and unmethylated probes) was used to detect the differential methylation of each CpG-site, whereas the Beta-value (rescaled between 0 and 1) was used for visualization (Du et al., 2010). The differential analysis of methylation data is similar with expression microarray data. We applied routines implemented in the LIMMA package (Smyth, 2004) to fit linear models to the normalized M-values. To ensure the selected CpG-sites had both high statistical significance and strong biological effects, we identified differentially methylated CpG-sites based on the following criteria: false-discovery-rate-adjusted (FDR-adjusted) p-value (q value) < 0.05 and an absolute DNA methylation fold-change > 2.00 (Because M-value is defined in the log₂ scale, the fold-change is defined as $2^{M_t - M_c}$, where M_t and M_c be the average methylation levels of pesticide-treated and control groups). All analyses were based on the mean of biological triplicates. CpG-sites with significantly different

methylation levels were then mapped to the closest downstream genes that may be affected in terms of their expression levels. Further functional enrichment analysis was performed for these overlapped genes based on Gene Ontology (GO) using the Bioconductor GeneAnswers package (Feng et al., 2010).

2.4. Pyrosequencing Verification

Two genes were examined, including aristaless-like homeobox 4 (ALX4) and O-6-methylguanine-DNA methyltransferase (MGMT), using pyrosequencing to validate our genome-wide DNA methylation data. The selection of these genes was based on statistical significance and biological function. PCR reactions were carried out using the Hotstart Taq polymerase kit (Qiagen, Valencia, CA) in a total volume of 25 μ L and with 50 pm of forward primer and reverse primer. For each PCR reaction, 50 ng of the bisulfite-converted DNA was used as a template. Bisulfite modification of genomic DNA was performed using an EZ DNA Methylation Gold kit according to the manufacturer's instructions (Zymo Research, Orange, CA). Pyrosequencing was performed using the PyroMark MD Pyrosequencing System (Biotage, Charlottesville, VA) as described previously (Xie et al., 2009). Methylation quantification was performed using the manufacturer's software. All samples were run in triplicate. The primers used in the PCR runs and pyrosequencing reactions are shown in Supplemental Table 2.

3. Results

3.1. Distinct methylation patterns of diazinon-treated cells

An overview of the sample relations based on heatmap of genome-wide DNA methylation profiles showed distinct methylation patterns of diazinon-treated cells in comparison with control cells (Figure 1). We identified 1069 CpG sites in 984 genes (918 hyper- and 66 hypomethylated genes) with >2-fold methylation changes in response to exposure of diazinon at 0.1 μ M. Our further analysis on gene functions by GO analysis showed that some of these genes are implicated in cancer development or in the related biological pathways, and some are functionally unknown (Table 1).

3.2. Hypermethylation of tumor suppressor genes

Among the 984 genes, several key tumor suppressor genes exhibit promoter in cells treated with diazinon. For example, diazinon-treated cells exhibited increased DNA methylation levels in genes of Ras-Association Domain Family 1 (RASSF1A) (3.0-fold, q-value=0.012), phosphatase and tensin homolog (PTEN) (2.6-fold, q-value<0.001), and early growth response 1 (EGR1) (4.4 fold, q-value<0.001). Other tumor suppressor genes with increased DNA methylation include putative tumor suppressor FUS2 (NAT2) (2.9-fold, q-value<0.001), candidate tumor suppressor in ovarian cancer 2 (OVCA2) (3.8-fold, q-value<0.001), p53-induced protein (TP53I11) (3.0-fold, q-value<0.001), and tumor protein p73 (TP73) (3.5-fold, q-value=0.005) (Table 1).

3.3. Methylation alteration in cancer-related genes

Some genes with biological functions related to cancer etiology, such as proliferation and apoptosis, DNA repair, telomere maintenance, and histone modification were also identified as having altered methylation levels. For example, several histone-related genes showed changes of methylation in response to exposure to diazinon, including H2A histone family; member O (HIST2H2AA) (-6.9-fold, q-value=0.009), H3 histone family; member A (HIST1H3A) (2.8-fold, q-value=0.008), histone deacetylase 3 (HDAC3) (2.2-fold, q-value=0.002) and histone mRNA 3 end-specific exonuclease (THEX1) (2.2-fold, q-value=0.007). Other genes include telomere-related genes, such as death-associated protein

6 (DAXX) (2.2-fold, q -value=0.005) and regulator of telomere elongation helicase 1 isoform 1 (RTEL1) (4.0-fold, q -value=0.009). Aberrant promoter methylation of proliferation-related genes has also been detected, such as mitogen-activated protein kinase 11 (MAPK11) (4.0-fold, q -value<0.001) and corticotropin releasing hormone receptor 1 (CRHR1) (3.7-fold, q -value=0.002) (Table 1).

3.4. Methylation alteration in genes with unknown function

In addition, we also observed genes/loci with unknown biological functions that exhibited significant fold changes in methylation level. A series of hypothetical protein showed large fold increases in DNA methylation level, such as hypothetical protein LOC146540 (146540) (10.7-fold, q -value=0.013), LOC203062 (TSNARE1) (9.5-fold, q -value<0.001), and LOC79979 (CXorf34) (8.9-fold, q -value<0.001) (Table 1).

3.5. Pyrosequencing of ALX4 and MGMT

These findings based on genome-wide analysis were validated by pyrosequencing using two selected genes. Pyrosequencing results for three of the selected genes were highly correlated with the genome-wide findings, including ALX4 ($R^2 = 0.85$) and MGMT ($R^2 = 0.84$) (Supplemental Figure 3).

4. Discussion

This is the first study on DNA methylation alteration in diazinon-treated human cells using the array-based genome-wide site-specific Illumina HumanMethylation27 platform. The Infinium DNA methylation platform is highly suitable for novel DNA methylation marker discovery. Although the methylation site coverage of the Illumina HumanMethylation27 platform is moderate and some available platforms have higher methylation site coverage, it has several advantages: 1) quantitative results (i.e., a precise measure reflecting percent methylation at any given methylation site); 2) single site resolution, unequivocally measuring methylation of 27,578 individual methylation sites; 3) being based on bisulfite treatment, a highly standardized preprocessing that ensures high reproducibility. In addition, we applied stringent statistical criteria to screen the CpG sites with expected FDR smaller than 5%.

Compared to controls, distinct genome-wide DNA methylation patterns were observed in relation to exposure to diazinon. In diazinon-treated cells, we identified 1069 CpG sites with significant methylation changes in 984 genes (918 hyper- and 66 hypomethylated genes). Gene ontology analysis demonstrated that some of these genes are tumor suppressor genes directly implicated in carcinogenesis, some have functions related to cancer etiology, and some remain functionally unknown (Table 1).

Diazinon has been associated with various cancers (Table 2), including leukemia (Beane Freeman et al., 2005), non-Hodgkin's lymphoma (Cantor et al., 1992; Waddell et al., 2001; Zahm et al., 1993), lung (Alavanja et al., 2004; Beane Freeman et al., 2005; Pesatori et al., 1994), brain (Davis et al., 1993) and prostate cancers. Aberrant tumor suppressor gene promoter methylation has emerged as one of the most important epigenetic mechanisms in the development of human cancers (Jones and Baylin, 2002). Altered DNA methylation of several cancer-related genes in response to diazinon was observed including 3-fold increases in the DNA methylation levels of RASSF1A and TP53I11. Inactivation of RASSF1A due to aberrant hypermethylation within promoter region is a frequent event in lung cancer patients (Fujiwara et al., 2005; Hsu et al., 2007). Promoter hypermethylation of p53 has been observed in individuals with leukemia (Agirre et al., 2003a; Agirre et al., 2003b; Martinez-Delgado et al., 2002), lung (Woodson et al., 2001) and brain tumors (Palani et al., 2010).

Exposure to Arsenic, which has been used as pesticide, was associated with hypermethylation at the promoter region of RASSF1A and p53 both *in vitro* and *in vivo* (Ren et al., 2011).

Several histone family genes and telomere-related genes showed altered methylation level in diazinon-treated cells, such as HIST1H3A, HIST2H2AA, and TREL1. Histone modification could alter their interaction with DNA and proteins (Kouzarides, 2007), and plays an important role in cancer development (Egger et al., 2004; Zhang and Dent, 2005). Telomere has been postulated to play a causal role in carcinogenesis by instigating chromosomal instability, thus promoting neoplastic transformation (Blasco, 2005). RTEL1 is an essential helicase for telomere maintenance and DNA repair (Barber et al., 2008; Ding et al., 2004; Youds et al., 2010). In the absence of RTEL1, telomeres are not maintained and chromosome fusions are observed (Uringa et al., 2011). Hypermethylation of RTEL, HIST1H3A and hypomethylation of HIST2H2AA observed in the present study suggest that diazinon may exert their effects by altering DNA methylation patterns, which could impact histones and telomeres.

Deregulated cell proliferation and apoptosis provide the underlying platform for neoplastic progression. These DNA methylation alterations may induce a wide range of potential abnormalities in gene expression patterns that can lead to dysregulation of processes related to cell transformation and tumorigenesis, including DNA repair, cell cycle control, genome stability and genome reprogramming (Jones and Baylin, 2007; Laird, 2005). Mitogen activated protein kinases (MAPKs), and Cyclin-dependent kinase inhibitor 1C (CDKN1C) all exhibited more than 2-fold increased methylation in response to diazinon. MAPKs are signal transducing enzymes implicated in a number of intracellular regulation events, such as proliferation, differentiation, stress response, and gene expression (Kim and Choi, 2010; Pearson et al., 2001). Cyclin-dependent kinases (CDK) are a family of enzymes that regulate the mammalian cell cycle and aberrant upregulation can lead to oncogenic effects (Larson et al., 2008). CDKN1C methylation associated with the gene down-regulation is observed in several cancers including lung cancers (Kobatake et al., 2004; Pateras et al., 2006). Activity of acetylcholinesterase (AChE) and cholinesterase, known biomarkers of toxicity of pesticide exposure (Tinoco-Ojanguren and Halperin, 1998), have been shown to decrease in occupational workers (Singh et al., 2011), farmers (Catano et al., 2008), and populations (Browne et al., 2006; Jintana et al., 2009) exposed to pesticides, leading to accumulation of acetylcholine (ACh). Sabunciyani et al. recently observed increased *PRIMA1* DNA methylation level in brain tissues of disorder patients with decreased AChE activity (Sabunciyani et al., 2012). OP-induced stress evoked a mutated AChE form called AChE-R, which was demonstrated to facilitate cellular metabolic activity and resistance to genotoxic stress (Mor et al., 2008). In addition to activity inhibition and mutation of AChE and cholinesterase, OPs exhibit adverse effect by modulation of the ACh receptors (AChR) (Smulders et al., 2004). Paliwal et al. showed the *CHRNA3* gene encoding the nicotinic AChR $\alpha 3$ subunit is a frequent target of aberrant DNA hypermethylation in lung cancer (Paliwal et al., 2010). These pieces of evidence suggest that ACh, AChR, AChE, and cholinesterase may be involved in the OP-induced methylation mechanisms. Furthermore, Zhang XJ et al. demonstrated that AChE may significantly reduce apoptosis in favor of proliferation (Jiang and Zhang, 2008; Zhang and Greenberg, 2012), which may promotes tumor progression. Further studies are needed to study the non-classical function of AChE in apoptosis and control of cell growth.

Pesticides may affect DNA methylation through several cellular processes, including oxidative stress/reactive oxygen species generation (Fratelli et al., 2005; Yu et al., 2008) and immunotoxicity (Daniel et al., 2001; Galloway and Handy, 2003). Diazinon has been shown to induce oxidative stress in animal studies. DNA methylation reflects the cumulative

oxidative stress, and ROS production has recently been shown to alter the expression of genes belonging to DNA methylation machinery (Fratelli et al., 2005). There is evidence supporting that the carcinogenesis from diazinon exposure may occur from decreased immunosurveillance (Galloway and Handy, 2003), such as decrease in total immunocyte count, immunocyte viability host resistance, and total IgG or IgM (Barnett et al., 1980; Booth and O'Halloran, 2001; Dutta et al., 1997; Khalaf-Allah, 1999). Chromatid exchange, a marker of chromosomal damage, has been shown in cell and human exposed to diazinon (Hatjian et al., 2000; Matsuoka et al., 1979). A dose-response genotoxic effect was observed in human cells treated with diazinon (Tisch et al., 2002). Despite the lack of clear evidence for carcinogenicity of pesticides, these cellular processes have been postulated to induce abnormalities in gene expression patterns, which in turn could lead to tumorigenesis.

There are a few limitations worthy of noting. We used K562 cells, a chronic myelogenous leukemia cell line, which resembles multi-potential hematopoietic cells. Therefore, our results should be interpreted with cautions. However, published studies by other researchers using K562 cells have demonstrated that DNA methylation can be altered by treatment with agents such as interleukin-6 and cadmium, suggesting that the K562 cell line is suitable for studying whether pesticides can induce DNA methylation alterations. Furthermore, because we also used the K562 cell line for the control group, our findings have demonstrated for the first time that three OPs have induced DNA methylation changes that were not induced in cells that were not exposed to OPs. Further studies will be required to determine if similar alterations in DNA methylation patterns would be observed in other cell types after exposure to these pesticides.

5. Conclusion

Our results provided direct experimental evidence that diazinon that has been associated with cancer risks in humans modify DNA methylation in promoter CpG sites of genes, many of which are carcinogenesis-related. The presented data, coupled with recent human epidemiology evidence linking pesticides with cancers, and that DNA methylation alterations as a hallmark of cancer, highlight the significance of the current study and provide mechanistic insights for further studies. In conclusion, the results from this investigation provide important data that pesticide exposure induces alterations in DNA methylation patterns. Further studies in different cell lines and *in vivo* samples are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CpG	Cytosine-Phosphate-Guanine
EPA	Environmental Protection Agency
FDR	False Discovery Rate
GO	Gene Ontology
OP	Organophosphate Pesticides

TL	Telomere Length
PCA	Principal Component Analysis
SSN	Simple Scaling Normalization

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Highlights

- We did a genome-wide examination of DNA methylation alterations in response to diazinon *in vitro*.
- 1069 CpG sites with significant methylation changes in 984 genes were observed.
- Some genes are implicated in cancer development or in cancer-related pathways.
- Diazinon may cause cancer via epigenetic mechanisms, such as DNA methylation alternation.

Figure 1. Distinct methylation pattern of Diazinon compared to control cells

A heatmap of all differentially methylated genes showed distinct methylation patterns of diazinon - treated cells in comparison with control cells. The heatmap color corresponds to the Beta - value of the measured CpG - sites. The Beta - value is in the range of 0 (shown in green) and 1 (shown in red) with 0 representing purely unmethylated and 1 representing purely methylated. The color bar above the heatmap represents the sample types, in which the red color represents diazinon - treated samples and black corresponds to the control samples. 0, 1, and 2 represent triplicate runs for each sample. 0, 1, 2, 3, 4 and 5 represent six runs for control.

Table 1

Selected genes with significant changes in methylation level

Symbol	Gene description	Entrez ID	Fold	q-value	Function
1. Selected Tumor suppressor genes					
EGR1	early growth response 1	1958	4.4	<0.001	positive regulation of transcription, DNA-dependent
OVC42	Candidate tumor suppressor in ovarian cancer 2	124614	3.8	<0.001	alydrolase activity
TP73	tumor protein p73	7161	3.5	0.005	DNA damage response; cell cycle arrest
TSSC4	tumor suppressing subtransferable candidate 4	10078	3.4	0.002	cellular Component
KISS1	KISS-1 metastasis-suppressor	3814	3.1	<0.001	negative regulation of cell proliferation
RASSF1A	Ras-Association Domain Family 1	11186	3.0	0.012	intracellular signal transduction; cell cycle arrest
TP53I11	p53-induced protein	9537	3.0	<0.001	negative regulation of cell proliferation; response to stress
NAT6	putative tumor suppressor FUS2	24142	2.9	<0.001	metabolic process
NBL	neuroblastoma; suppression of tumorigenicity 1 precursor	4681	2.7	0.007	positive regulation of neuron differentiation
PTEN	phosphatase and tensin homolog	5728	2.6	<0.001	prostate gland growth; negative regulation of cell proliferation
2. Selected Cancer-related genes					
<u>a) Telomere-related genes</u>					
RTEL1	regulator of telomere elongation helicase 1 isoform 1	51750	4.0	0.009	telomere maintenance; DNA repair
DAXX	death-associated protein 6	1616	2.2	0.005	Induction of apoptosis
<u>b) Histone-related genes</u>					
HIST2H2AA	H2A histone family; member O	8337	-6.9	0.009	nucleosome assembly; DNA binding
HIST1H3A	H3 histone family; member A	8350	2.8	0.008	Regulation of gene silencing
HDAC3	histone deacetylase 3	8841	2.2	0.002	histone deacetylation; chromatin modification
THEX1	histone mRNA 3 end-specific exonuclease	90459	2.2	0.007	histone mRNA catabolic process
<u>c) Proliferation/apoptosis-related genes</u>					
CDC20	cell division cycle 20	991	5.3	<0.001	cell cycle checkpoint; positive regulation of cell proliferation
MAPK11	mitogen-activated protein kinase 11	5600	4.0	<0.001	response to stress; intracellular protein kinase cascade
CDKN1C	cyclin-dependent kinase inhibitor 1C	1028	3.8	<0.001	negative regulation of cell proliferation; cell cycle arrest
IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1	10642	3.3	0.016	regulation of mRNA stability involved in response to stress
GAS2L1	growth arrest-specific 2 like 1 isoform a	10634	3.0	0.005	cell cycle arrest
GAS6	growth arrest-specific 6	2621	2.8	<0.001	regulation of growth

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Symbol	Gene description	Entrez ID	Fold	q-value	Function
CDKN1A	cyclin-dependent kinase inhibitor 1A	1026	2.4	0.011	cell cycle arrest; cellular response to extracellular stimulus
CDK4	cyclin-dependent kinase 4	1019	2.1	0.002	regulation of cell cycle; positive regulation of cell proliferation
P53AIP1	p53-regulated apoptosis-inducing protein 1	63970	2.2	<0.001	regulation of apoptosis
d) Other genes					
ALX4	aristaless-like homeobox 4	60529	4.3	<0.001	regulation of apoptosis
CRHR1	corticotropin releasing hormone receptor 1	1394	3.7	0.002	epithelial cell differentiation; immune response
ILF3	interleukin enhancer binding factor 3 isoform a	3609	3.6	0.012	negative regulation of transcription, DNA-dependent
ST3GAL2	sialyltransferase 4B	6483	3.2	<0.001	protein modification process
UVRAG	UV radiation resistance associated gene	7405	2.9	<0.001	DNA repair
NAT10	N-acetyltransferase-like protein	55226	2.1	0.001	N-acetyltransferase activity; metabolic process
3. Selected genes with unknown functions					
FLJ32130	hypothetical protein LOC146540	146540	10.7	0.013	N/A
TSNARE1	hypothetical protein LOC203062	203062	9.5	<0.001	N/A
CXorf34	hypothetical protein LOC79979	79979	8.9	<0.001	N/A

Genes were sorted by absolute fold change

Table 2
Profile of diazinon

Characteristic	Information
Chemical name	O,O-Diethyl O-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate
Chemical formula	C12H21N2O3PS
Chemical structural
Toxicity classification	Category III- slightly toxic
EPA group	Group D-not classifiable as to human carcinogenicity

Association with cancer	Species	Reference
leukemia	Human	(Beane Freeman et al., 2005)
NHL	Human	(Cantor et al., 1992; Waddell et al., 2001; Zahm et al., 1993)
lung	Human	(Alavanja et al., 2004; Beane Freeman et al., 2005; Pesatori et al., 1994)
prostate cancers	Human	(Band et al., 2011)
brain	Human	(Davis et al., 1993)

Toxic effects		
immunotoxicity	Animal	(Barnett et al., 1980; Booth and O'Halloran, 2001; Dutta et al., 1997; Galloway and Handy, 2003; Handy et al., 2002; Khalaf-Allah, 1999)
genotoxicity	<i>In vitro</i>	(Tisch et al., 2002)
chromosomal damage	<i>In vitro</i> and Human	(Hatjian et al., 2000; Matsuoka et al., 1979)