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

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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.



*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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## 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, methoxyclor, 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 h). 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 (Supplementary Table 1) or show cytotoxic effects (Supplementary Fig. 1), and are similar to exposure levels experienced by pesticide applicators in real life (Arcury et al., 2007, 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 25 ng/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 0.1 μM for 12 h using Illumina Infinium HumanMethylation27 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