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Aberrant Hypomethylated KRAS and RASGRF2 as a Candidate Biomarker of Low Level Benzene Hematotoxicity

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Abstract

Benzene is an important industrial chemical and an environmental contaminant. The mechanisms of low level benzene-induced hematotoxicity are unresolved. Aberrant DNA methylation, which may lead to genomic instability and the altered gene expression, is frequently observed in hematological cancers. The purpose of the present study was to conduct a genome-wide investigation to examine comprehensively whether low level benzene induces DNA methylation alteration in the benzene-exposed workers. Infinium 450K methylation array was used to compare methylation levels of the low level benzene-exposed individuals and health controls and the differentially expressed DNA methylation pattern critical for benzene hematotoxicity were screened. Signal net analysis showed that two key hypomethylated KRAS and RASGRF2 associated with low level benzene exposure were identified. Further, the hypomethylated RASGRF2 gene played central roles through regulation of Rho protein, MAPK, small GTPase mediated signal transduction. While the hypomethylated KRAS gene played important roles through small GTPase, Ras protein, MAPK cascade, Gap junction, Axon guidance, Tight junction, GnRH, T cell receptor signaling pathway, Acute myeloid leukemia, B cell receptor signaling pathway, Chronic myeloid leukemia, ErbB signaling pathway. Our preliminary study indicated that aberrant hypomethylated KRAS and RASGRF2 might be a potential methylated biomarker of low level benzene hematotoxicity.

Keywords: Low level benzene exposure; Biomarkers; DNA methylation; KRAS; RASGRF2

Introduction

Benzene is an important industrial chemical and an environmental contaminant. Chronic occupational benzene exposure is associated with an increased risk of hematological malignancies. A study indicated that white blood cell counts were decreased in workers exposed to <1 ppm benzene compared with controls [1]. However, the mechanisms of low level benzene-induced hematotoxicity remain to be elucidated.

DNA methylation alteration is very useful in the diagnosis, prognosis and prediction of disease [2]. Methylation of 5'-CpG islands in gene promoter regions has consistently been found in malignant tissues and is hypothesized to be indicative of critical early changes in cancer development [3]. Aberrant DNA methylation, which may lead to genomic instability and the altered gene expression, is frequently observed in AML [4]. Repeated-element DNA hypomethylation, as well as gene-specific hypermethylation or hypomethylation are commonly seen in hematological cancers [5]. DNA methylation has thus been proposed to reflect environmentally-induced epigenetic reprogramming and risk of future disease [6-8].

Benzene, an established human leukemogen, is known to induce global DNA hypomethylation changes through its active metabolites including hydroquinone (HQ) in human TK6 lymphoblastoid cells [9]. Global DNA hypomethylation was reported based on PCR-Pyrosequencing measures in gasoline station attendants exposed to benzene [10] and these studies suggest that DNA hypomethylation may be key mechanism underlying the leukemogenicity of benzene. However, knowledge of the effects of benzene on DNA methylation is still limited. Therefore, we hypothesis that low level benzene exposure can induce population-specific methylation alterations in the promoter regions of specific genes, thus leading to changes in gene transcription and an increased risk for benzene hematotoxicity. To gain an insight into the molecular mechanisms and new biomarkers, microarray analysis was used to identify the differentially expressed DNA methylation pattern critical for benzene hematotoxicity. Specific CpG sites (or genes) with altered methylation in workers due to exposure to low level benzene were identified.

Materials and Methods

Characteristics of subjects

These benzene-exposed subjects are all paint sprayers and health controls are office workers without benzene exposure in the same factory. The detailed characteristics of study population can be seen in our previous published papers [11,12]. Air benzene exposure was determined by a passive sampler (stainless steel tube, internal diameter of 9 mm, length of 90 mm) containing Chromosorb 106, worn by the study subjects near the breathing zone during the work shift. Benzene was determined by thermal desorption followed by gas chromatography/flame ionization detector analysis [10,13]. Airborne benzene concentration between health controls without benzene exposure and low level benzene-exposed group without clinical symptoms is 0.06 ± 0.01mg/m^3, 1.82 ± 1.16 mg/m^3, respectively.

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Each subject was given a physical exam by a study physician. A questionnaire was administered to obtain the detailed information on occupation, environmental exposures to solvents and pesticides, past and current tobacco, alcohol use and medical history including recent infections, ionizing radiation exposure, medication use and family history. The subjects in each group were matched by age, gender and life styles. These samples were used for microarray analysis. Briefly, peripheral blood samples were randomly obtained from four benzene-exposed workers (average age 44.7 (39~51) yrs, average white blood cell $6.4*10^9$/L) and four health controls without benzene exposure (average age 43.3(34~55) yrs, average white blood cell $6.4*10^9$/L) with informed consents, and the study was approved by the Committees for Ethical Review of Research involving Human Subjects of Capital Medical University.

Peripheral blood genomic DNA isolation and bisulphite conversion

Genomic DNA from the peripheral blood mononuclear cells of four workers exposed to benzene levels ranging < 1 ppm and four health controls was isolated by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). DNA was prepared with a Wizard Genomic DNA purification kit (Promega Corp, Madison, WI), quantified, and diluted into aliquots of 25 ng/μl for genome-wide DNA methylation analysis. One μg of genomic DNA was treated using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Unmethylated cytosines were converted to uracils (which are then converted into thymines following subsequent PCR), while methylated cytosines remained unchanged. We used a total of 500 ng DNA for each bisulphite conversion reaction. Bisulfite treated DNA was aliquoted and stored at −80°C until ready for use.

Infinium DNA methylation analysis

The 450K DNA Methylation array includes 485,764 cytosine positions of the human genome. A general depiction of the 450K platform design, regarding functional genome distribution, CpG content and chromosome location, is reported and validated in two independent laboratories [14,15]. Genome-wide methylation analysis was performed using the high resolution Infinium 450K methylation array interrogating about 480 000 CpGs distributed in promoters, gene bodies, 3'UTRs and intergenic regions [15]. DNA methylation data were processed using GenomeStudio software (ver.2012; Illumina, Inc.) applying the default settings. Infinium arrays were hybridized and scanned as manufacturer's instructions (Illumina, San Diego, USA). Individual probe β values (range 0-1) are approximate representations of the absolute methylation percentage of specific CpG sites within the sample population. Beta (β)=1 indicates complete methylation; β=0 represents no methylation. The values are derived by comparing the ratio of intensities between the methylated and unmethylated alleles using the following formula: β value=Max (Signal B, 0)/[Max (Signal A, 0) + Max (Signal B, 0)]. Where Signal B is the array intensity value for the methylated allele and Signal A is the non-methylated allele. Samples were processed using the Bioconductor package, which is specifically designed for Illumina data. All the obtained DNA methylation data have been deposited in the Gene Expression Omnibus (GEO) database in the following link: acc=GSE50967

Hierarchical clustering

To ascertain whether these differentially expressed hypomethylated and hypermethylated genes among groups were selected correctly, hierarchical cluster analysis was performed based on differentially expressed genes using Cluster Treeview software from Stanford University.

Differentially expressed hypomethylated and hypermethylated genes were identified through random variance model (RVM)

Because of high test fee, only four samples at each group were detected. The age, gender, lifestyle such as smoking, drinking, and medical history in each group were matched to reduce the impact of these confounding factors. In addition, the effective statistical method for small samples was adopted. RVM, t-test was commonly used for comparison of two groups, because the RVM, t-test effectively increased the degrees of freedom in the cases of small samples [16,17]. After the significance analysis and false discovery rate (FDR) analysis, we selected the differentially expressed genes according to the statistical significance of t-test after adjustment with the Benjamini and Hochberg correction. The cut-off for t-test P-values is <0.001.

Gene ontology analysis and pathway analysis

Gene ontology analysis was applied to analyze the main function of differential expression genes according to the Gene ontology project [18]. Fisher's exact test and χ^2 test were used to classify the GO category, and the FDR was calculated to correct the *P* value [19]. The standard of difference screening was $P<0.05$. Similarly, pathway analysis was used to find out the significant pathway of the differential genes according to KEGG, Biocarta and Reatome. Fisher's exact test and χ^2 tests were used to select the significant pathway, and the threshold of significance was defined by *P* value and FDR [20-22]. The standard of difference screening was $P<0.05$.

Signal-Net analysis

Gene-gene interaction network was constructed based on the data of differentially expressed mRNAs. For instance, if there is confirmative evidence that two genes interact with each other, an interaction edge is assigned between the two genes. The considered evidence is the source of the interaction database from KEGG. Networks are stored and presented as graphs, where nodes are main genes (protein, compound, etc.) and edges represent relation types between the nodes, e.g. activation or phosphorylation. The graph nature of networks raised our interest to investigate them with powerful tools implemented in R [23]. To investigate the global network, we computationally identify the most important nodes. To this **e**nd we turn to the connectivity (also known as degree) defined as the sum of connection strengths with the other network genes: $K_i = \sum_{u \neq i} a_{ui}$. In gene networks, the connectivity measures how correlated a gene is with all other network genes. For a gene in the network, the number of source genes of a gene is called the indegree of the gene and the number of target genes of a gene is its outdegree. The character of genes is described by betweenness centrality measures reflecting the importance of a node in a graph relative to other nodes. For a graph G:(V,E) with n vertices, the relative betweenness centrality $C'_B(v)$ is defined by: $C'_B(v) = \frac{2}{n^2 - 3n + 2} \sum_{s \neq v \neq t \in V} \frac{\sigma_{st}(v)}{\sigma_{st}}$ where $\sigma_{st}(v)$ is the number of shortest paths from s to t, and $\sigma_{st}(v)$ is the number of shortest paths from s to t that pass through a vertex v. [24-26]. We were thus able to search for differential expression genes. Two nodes were connected when their corresponding encoded gene products were either directly connected or indirectly connected by a linker gene in the interaction network. The network for each gene was measured by counting the number of upstream and downstream genes or binding genes which were expressed as in-degree and out-degree or degree, respectively. The higher degree of a gene indicates that it is

regulating or being regulated by a greater number of genes, implying it has a more important role in the signaling network. $P<0.05$ was considered statistically significant. Gene interactions could then be drawn based on the data [27].

Results

The differentially hypomethylated and hypermethylated CpG pattern associated with low level benzene exposure and functional analysis

Distinct genome-wide DNA methylation patterns were observed in benzene exposures workers compared with health controls. Hierarchical cluster analysis shows 1546 differential hypermethylated and 613 hypomethylated CpG sites in low level benzene exposure workers compared to health controls (Figure 1a). GO analysis indicates that these hypermethylation genes are mainly involved in cell adhesion, signal transduction, transcription, DNA-dependent, regulation of small GTPase mediated signal transduction, apoptotic process, cell differentiation, positive regulation of cell proliferation, G-protein coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger, oxidation-reduction process, Wnt receptor signaling pathway, cellular response to oxidative stress, innate immune response, induction of apoptosis, immune response, JUN phosphorylation, regulation of JUN kinase activity, leukemia inhibitory factor signaling pathway (Figure 1b); While these hypomethylation genes in low level benzene exposure workers are implicated in transcription, DNA-dependent, small GTPase mediated signal transduction, cell adhesion, Ras protein signal transduction, synapse assembly, negative regulation of transcription, DNA-dependent, MAPK cascade, DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest, post-translational protein modification, protein autophosphorylation, signal transduction, negative regulation of cell proliferation, negative regulation of NF-kappaB import into nucleus, negative regulation of JAK-STAT cascade, cell differentiation (Figure 1c).

Genomic distribution of the differentially methylated CpGs sites associated with low level benzene exposure with respect to functional genomic distribution (promoter, gene body, 3'UTR and intergenic) and CpG content (CpG island, shore, shelf and open sea)

Our preliminary study identified a set of differentially methylated CpG loci between low level benzene exposure workers and health controls. The location of differentially methylated CpG loci with respect to CpG content and functional genomic distribution was similar in our study, the majority of CpG loci were residing in gene promoters (Figure 2a) and in CpG islands (Figure 2b).

Pathway analysis of the differential methylation genes associated with low level benzene exposure

To further investigate key pathways linked to these distinct genes,

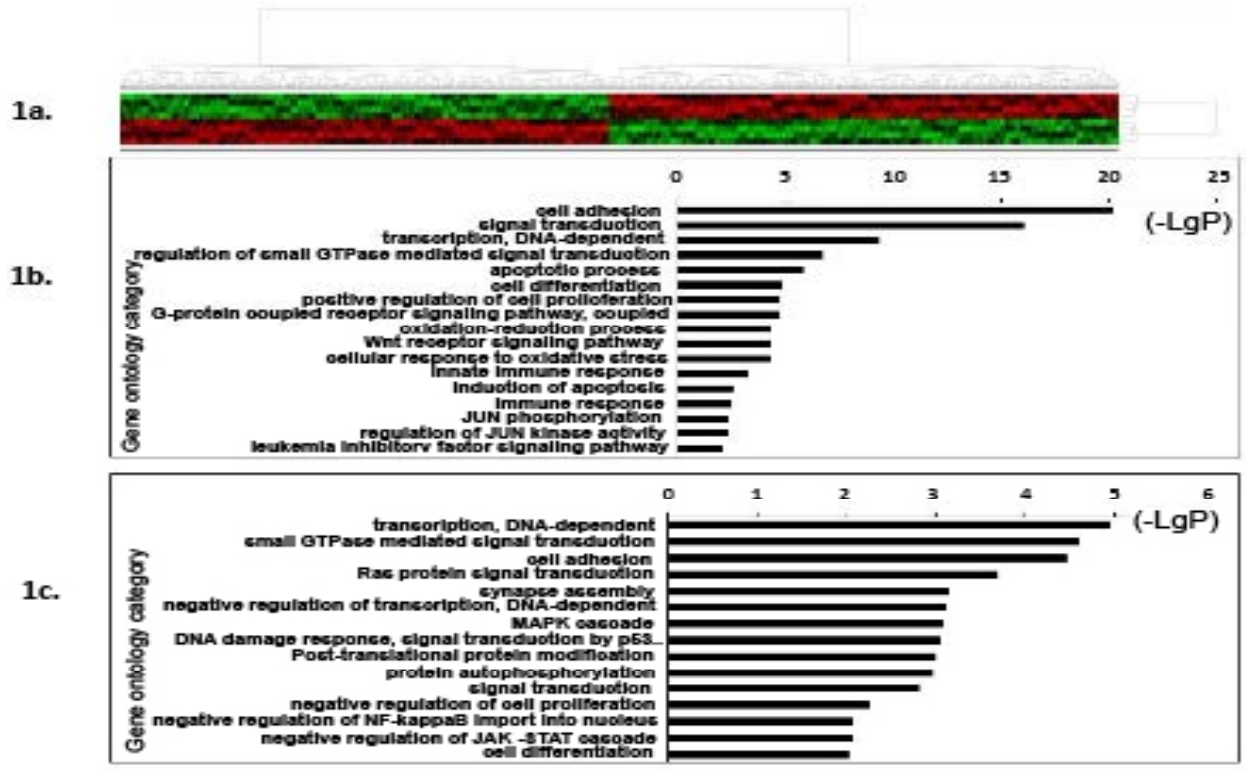

Figure 1: The differentially hypomethylated and hypermethylated CpG profiles associated with low level benzene exposure and functional analysis. (a) Hierarchical cluster of 2159 differentially methylated CpG sites in low level benzene exposure workers compared with health controls. Among them, there are 1546 differential hypermethylated and 613 hypomethylated CpG sites. 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. Each row represents a separate sample and each column represents a CpG site. (b) GO analysis of differential hypermethylated genes in low level benzene exposure workers compared to health controls. (c) GO analysis of differential hypomethylated genes in low level benzene exposure workers compared to health controls. X axis, negative logarithm of the P value (-LgP), the larger the value was, the smaller the P value was. Y axis, the name of the gene ontology category, significant GO terms of differential methylated genes.

the significant pathways categories (P<0.05) of differential genes associated with benzene exposure were performed. Our analysis showed that differentially hypomethylated genes were distributed in 12 significant pathways, including MAPK signaling pathway, Gap junction, Cell adhesion molecules (CAMs), p53 signaling pathway, Cytokine-cytokine receptor interaction, Notch signaling pathway, GnRH signaling pathway, T cell receptor signaling pathway, Acute myeloid leukemia, Mismatch repair, B cell receptor signaling pathway, Chronic myeloid leukemia (Table 1). While these differentially hypermethylated genes associated with low level benzene exposure were distributed in 14 significant pathways, including Cell adhesion molecules (CAMs), Cytokine-cytokine receptor interaction, Apoptosis, MAPK signaling pathway, Acute myeloid leukemia, Hematopoietic cell lineage, Oxidative phosphorylation, Notch signaling pathway, Wnt signaling pathway, Toll-like receptor signaling pathway, p53 signaling pathway, Adherens junction, Tight junction, ErbB signaling pathway (Table 2).

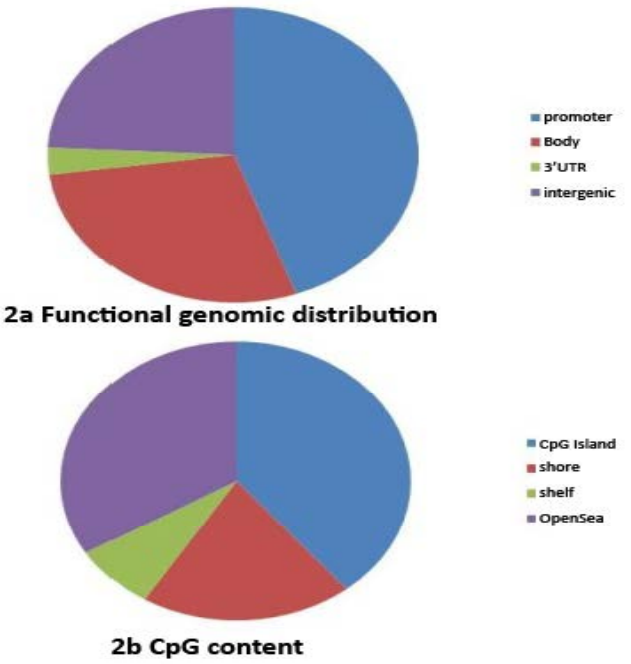

Figure 2: Genomic distribution of the differentially methylated CpGs sites in low level benzene exposure workers compared with health controls with respect to functional genomics distribution (promoter, gene body, 3′UTR and intergenic) and CpG content (CpG island, shore, shelf and open sea). The location of differentially methylated CpG loci with respect to CpG content and functional genomics distribution was similar in our study, with the majority of CpG loci residing in CpG islands and in gene promoters.

Pathway	p-Value	FDR	Gene Symbol
MAPK signaling pathway	6.40E-08	5.65E-06	MKNK2, CACNB4, EGF, HSPA1L, MOS, RASGRF2, MAPK8IP3, CEBPZ, HSPA1A, HSPA1A, KRAS, MAPKAPK5, PTPN7, TNF
Gap junction	1.09E-04	6.33E-04	GNA11, EGF, ADCY2, CDC2, CDK1, KRAS, PDGFD
Cell adhesion molecules (CAMs)	1.12E-04	6.33E-04	NRXN1, HLA-DQA2, HLA-F, HLA-DQA1, HLA-G, PDCD1, PVRL2
p53 signaling pathway	0.002349	0.005206	ATR, GTSE1, CDC2, CDK1, MDM2
Cytokine-cytokine receptor interaction	0.005546	0.010259	EGF, BMP7, FLT4, CCL14, RELT, TNF, TNFSF13B
Notch signaling pathway	0.006421	0.0117	NCOR2, CTBP2, JAG2
GnRH signaling pathway	0.011063	0.017584	GNA11, PLD1, ADCY2, KRAS
T cell receptor signaling pathway	0.011779	0.01834	PDCD1, CARD11, KRAS, TNF
Acute myeloid leukemia	0.011996	0.01856	JUP, KRAS, RUNX1T1
Mismatch repair	0.014568	0.021396	CDC2, MSH2
B cell receptor signaling pathway	0.022695	0.028704	CARD11, FCGR2B, KRAS
Chronic myeloid leukemia	0.022695	0.028704	CTBP2, KRAS, MDM2

Table 1: Pathway analysis of differentially hypomethylated genes associated with low level benzene exposure.

To find the interaction among pathways directly and systemically, the interaction net of the significant pathways associated with low level benzene exposure was built according to the KEGG database. As shown in Figure 3, key pathway interaction of differentially methylated genes associated with low level benzene exposure in this study were mainly involved in MAPK signaling pathway, p53 signaling pathway, Adherens junction, Cytokine-cytokine receptor interaction, Cell adhesion molecules (CAMs), ErbB signaling pathway. Interestingly, T cell receptor signaling pathway, Focal adhesion, B cell receptor signaling pathway were special pathways for differentially hypomethylated genes. However, apoptosis, Wnt signaling pathway, TGF-beta signaling pathway, calcium signaling pathway were special pathways for differentially hypermethylated genes.

Signal-net analysis of distinct methylated genes in low level benzene-exposed workers

To investigate the key genes involved in low level benzene exposure,

Pathway	p-Value	FDR	Gene Symbol
Cell adhesion molecules (CAMs)	3.27E-05	2.02E-04	HLA-DQB1, NFASC, NLGN4X, CD34, CDH2, MAG, MAL, VCAN
Cytokine-cytokine receptor interaction	1.57E-04	6.61E-04	TNFRSF8, IL17RB, KIT, BMPR2, CXCL1, CXCL6, IFNGR2, INHBC, TNFRSF11B, TNFRSF19
Apoptosis	1.64E-04	6.73E-04	BAD, MYD88, NGF, APAF1, CASP7, PRKAR2B
MAPK signaling pathway	9.92E-04	0.00264	CACNA1H, ECSIT, BDNF, NGF, CACNG4, DUSP10, DUSP3, JUN, MEF2C
Acute myeloid leukemia	0.00204	0.00477	TCF7L2, BAD, CCNA1, KIT
Hematopoietic cell lineage	0.008189	0.012519	KIT, CD34, CD7, MAL
Oxidative phosphorylation	0.008911	0.012955	NDUFV2, ATP6V0E2, ATP6V0A4, LOC642502, SDHC
Notch signaling pathway	0.008914	0.012955	LFNG, HES5, NUMB
Wnt signaling pathway	0.01276	0.016638	TCF7L2, FZD8, JUN, PRICKLE1, SFRP2
Toll-like receptor signaling pathway	0.014075	0.017825	TIRAP, MYD88, JUN, MAL, TLR2
p53 signaling pathway	0.02491	0.02784	TEP1, APAF1, CHEK2
Adherens junction	0.035213	0.034955	TCF7L2, ASIP, IQGAP1
Tight junction	0.035657	0.034955	TEP1, PRKCZ, ASIP, PARD6B
ErbB signaling pathway	0.0448	0.040811	BAD, NRG1, JUN

Table 2: Pathway analysis of differentially hypermethylated genes associated with low level benzene exposure.

signal-net analysis of differential methylated genes associated with low level benzene exposure showed that two key hypomethylated KRAS and RASGRF2 were identified according to the degree size (Figure 4 and Table 3). Further GO analysis indicated that the hypomethylated RASGRF2 gene played central roles through regulation of Rho protein signal transduction, MAPK signaling pathway, small GTPase mediated signal transduction, synaptic transmission (Supplemental material, Supporting Table S1) and through MAPK signaling pathway (Supplemental material, Supporting Table S2). While hypomethylated KRAS gene played important roles through small GTPase mediated

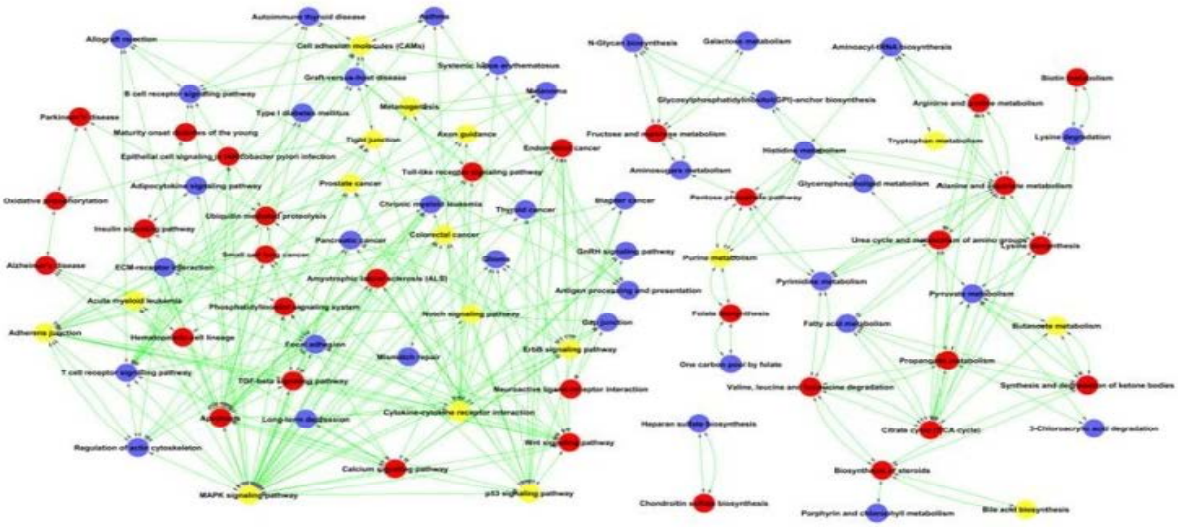

Figure 3: Pathnet analysis of differential methylation genes in low level benzene-exposed workers. Nodes represent pathways. The area of nodes displays the degree which is the number of other pathways that interact with this pathway. Lines indicate interactions between pathways, where pathways indicated by the arrow head are regulated by pathways of the arrow tail. Red denotes the hypermethylated pahtway. Blue denotes the hypomethylated pahtway. Yellow denotes the hypermethylated and hypomethylated pahtway.

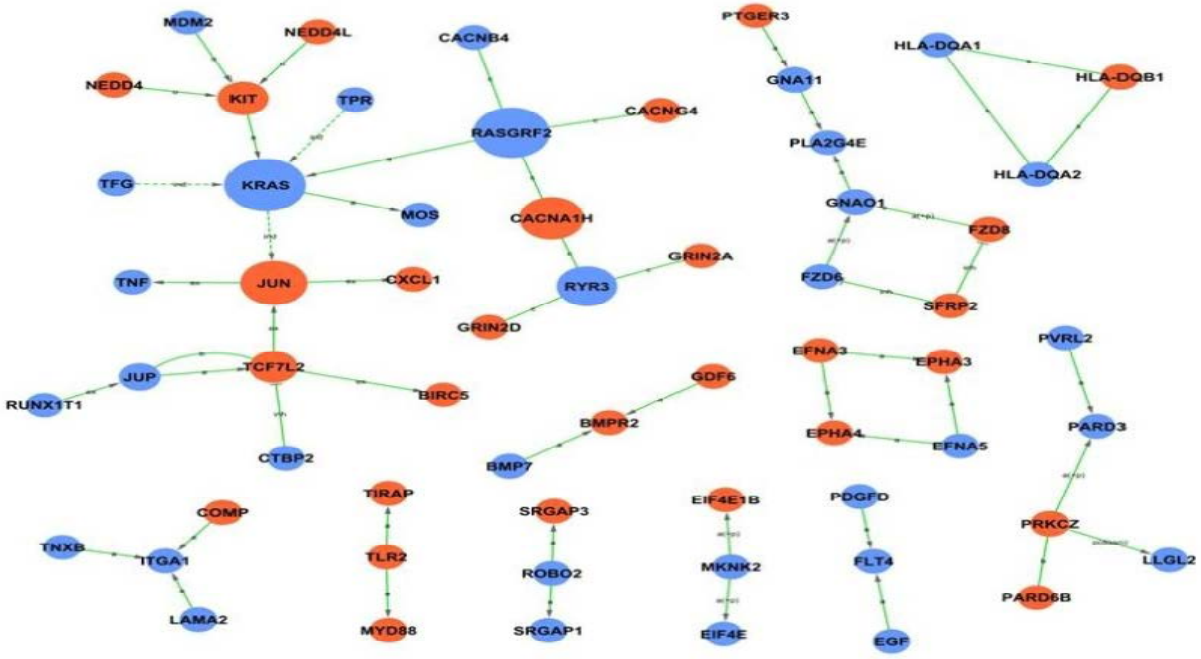

Figure 4: Signal-net analysis of differential methylated genes in low level benzene-exposed workers. The molecular networks were constructed, where nodes are main genes and edges represent relation types between the nodes, e.g. activation or phosphorylation. Red denotes the hypermethylated genes. Blue denotes the hypomethylated genes.

Gene symbol	Gene symbol Description	Betweenness centrality	Degree	Regulation stytle
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	0.0377	6	low
RASGRF2	Ras protein-specific guanine nucleotide-releasing factor 2	0.0348	4	low
JUN	jun proto-oncogene	0.0261	4	high
CACNA1H	calcium channel, voltage-dependent, T type, alpha 1H subunit	0.0239	2	high
RYR3	ryanodine receptor 3	0.020	3	low
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	0.010	4	high

The degree is defined as the sum of connection strengths with the other network genes. For a gene in the network, the number of source genes of a gene is called the indegree of the gene and the number of target genes of a gene is its outdegree. The character of genes is described by betweenness centrality measures reflecting the importance of a node in a graph relative to other nodes.

Table 3: The top 6 significantly methylated genes according to the degree size Supporting Information.

signal transduction, Ras protein signal transduction, MAPK cascade, axon guidance, blood coagulation (Supplemental material, Supporting Table S1) and through MAPK signaling pathway, Gap junction, Regulation of actin cytoskeleton, Axon guidance, Tight junction, GnRH signaling pathway, T cell receptor signaling pathway, Acute myeloid leukemia, B cell receptor signaling pathway, Chronic myeloid leukemia, Colorectal cancer, ErbB signaling pathway (Supplemental material, Supporting Table S2).

Discussion

In this study, the overall goal is to provide early methylated biomarkers of low level benzene exposure and to have an in-depth insight into the molecular mechanisms regulating benzene hematotoxicity. Our study indicated that aberrant hypomethylated KRAS and RASGRF2 might be a candidate methylation biomarker of low level benzene hematotoxicity. This study suggests that low level benzene may cause toxicity via non-mutagenicity mechanisms and benzene-induced toxicity may be mediated in part by epigenetic mechanisms.

Toxicogenomic studies of exposed population are an important alternative approach to the human health risk assessment of environmental exposures and have been used to identify potential biomarkers of early effects and mechanisms underlying associated diseases. Microarray techniques have been used successfully to monitor DNA methylation profile changes. The recent advances in methods to examine epigenetic modifications, such as DNA methylation, have led to interest in determining the genome-wide epigenetic profiles characterizing many different disease states. Various efficient technological platforms have been developed in recent years for high-throughput genome-wide analysis of DNA methylation. One of these is the Infinium 450K methylation array by Illumina [15]. The Infinium 450k DNA methylation array from Illumina offers the possibility to analyze more than 480,000 individual CpG sites in a user friendly standardized format [14,15]. This setup enabled us to unravel alteration in DNA methylation at high resolution independent of genetic variation. This platform has been successfully applied to detect aberrantly methylated genes [28,29]. Deneberg et al. derived genome-wide DNA methylation profiles of 29 childhood B-ALL patients and four normal B-cell samples using the Infinium 450 K DNA methylation Bead assay [30].

Compared with previously released Illumina DNA methylation platforms, the recently launched Infinium Human Methylation 450 BeadChip represents a significant increase in the CpG site density for quantifying methylation events. At the gene level, the 450K microarray covers 99% of RefSeq genes with multiple sites in the annotated promoter (1500 bp or 200 bp upstream of transcription start site), 5-UTR, first exon, gene body and 3-UTR. From the CpG context, it covers 96% of CpG islands with multiple sites in the annotated CpG Islands, shores (regions flanking island) and shelves (regions flanking shores) [15]. While the role of DNA methylation in promoter and CpG island regions has been appreciated, the importance of DNA methylation in gene body or shore regions for transcription regulation and tumor initialization has recently come to attention [31,32]. The significantly increased coverage makes 450K microarray a powerful platform for exploring methylation profile in these annotated regions. As each targeted region contains at least one CpG site, treating the region as a unit in the differential methylation analysis might help identify regions with consistently coordinate methylation changes. The promoter, 5-UTR, first exon, gene body and 3-UTR are gene-based regions. The CpG island and its surrounding shore and shelve regions are not necessary gene based, depending on their distance to the nearest genes. Our preliminary study indicated that the majority of differentially hypomethylated and hypermethylated CpG loci associated with low level benzene exposure were residing in gene promoters and in CpG islands. Our results provide direct experimental evidence that benzene, associated with increased cancer risk, cause changes in promoter CpG methylation in numerous genes.

DNA methylation is an essential epigenetic mark that is required for normal development. DNA methylation plays a vital role in transcriptional regulation and chromatin remodeling. The aberration of DNA methylation profile has been found to be associated with many human diseases including cancer. Use of DNA methylation microarray is a popular approach to characterize the epigenetic landscape of human cells [33]. DNA methylation alterations in gene promoters have also been found repeatedly following exposure to various environmental chemicals, including pesticides [34], phenol and hydroquinone [35] and dioxidine [36], suggesting that DNA methylation is inducible by chemical exposures.

We previously observed DNA methylation alterations in specific genes and our data showed effects of benzene on DNA methylation of specific genes such as PARP-1 and PTEN in cells and animal modes treated by benzene [37,38]. Epigenetic mechanisms of gene regulation are heritable, reversible modifications that are critical for the organization of chromatin and regulation of tissue-specific gene expression. DNA methylation is a dynamic epigenetic mark primarily localized to cytosine residues in the context of a CpG dinucleotide in mammals. While the critical role for DNA methylation in early development is clearly established, however the role for DNA methylation in peripheral blood mononuclear cells is less understood. In this study, we conducted genome-wide DNA methylation analyses on DNA samples obtained from the peripheral blood mononuclear cells of low level benzene exposure workers (< 1 ppm) and health controls using the high resolution Infinium 450K methylation array. To our knowledge, this is the first pilot study of genome-wide DNA methylation analyses for low level benzene exposure workers (<1 ppm) using the high resolution Infinium 450K methylation array. Thus, the use of noninvasive measurements

of peripheral blood samples could provide a highly feasible method to examine benzene hematotoxicity-associated epigenetic changes of certain genes. The methylation of the gene from peripheral blood cells was commonly associated with all kinds of diseases [36,39-41].

Hypermethylation of tumor suppressor p15, which leads to inactivation of the gene and subsequent uncontrolled proliferation of the cell, is commonly observed in AML and other hematological cancers [42-45]. A significant reduction in LINE-1and AluI methylation and hypermethylation in p15 and hypomethylation in MAGE-1 were associated with increasing airborne benzene levels [10]. Seow WJ et al showed statistically significant but weak associations of LINE-1 and p15 hypomethylation with SPMA in 158 Bulgarian petrochemical workers [46]. Carugno M et al found that low-dose benzene exposure was associated with increased mitochondrial DNA copy number. Benzene exposure may be associated with hypermethylation in ERCC3 [47]. The melanoma antigen family A (MAGE) gene, which encodes tumor rejection antigens, is widely expressed in cells from hematological malignancies and is found to be hypomethylated with benzene exposure [10,48]. All these results suggested that effect of benzene hematotoxicity may be mediated in part by DNA methylation which can substantially affect gene transcription without changing DNA sequence.

In this study, we investigated the association between occupational benzene exposures with gene specific DNA methylation in low level benzene exposures workers. Our study indicated that aberrant hypomethylated KRAS and RASGRF2 might be a candidate methylation biomarker of low level benzene hematotoxicity. Further GO and pathway analysis indicated that hypomethylated RASGRF2 gene played central roles through regulation of small GTPase mediated signal transduction. While hypomethylated KRAS gene played important roles through small GTPase mediated signal transduction, Ras protein signal transduction, T cell receptor signaling pathway, Acute myeloid leukemia, B cell receptor signaling pathway, Chronic myeloid leukemia, ErbB signaling pathway. Functional analysis of methylation changes revealed that differential methylation genes were involved in carcinogenesis-related processes. Our results provide experimental evidence that benzene exposures may modify gene promoter DNA methylation levels, suggesting that epigenetic mechanisms may contribute to benzene-induced carcinogenesis.

Aberrant methylation of promoter CpG that causes silencing of tumor suppressor genes (TSGs) may play a key role in the carcinogenesis of many cancer types. RASGRF2 has only been reported to possibly play a role in the pathogenesis of pancreatic cancer cell lines [48]. Frequent methylation and silencing of RASGRF2 in tumor cells may play an important role in the carcinogenesis of non-small cell lung cancer (NSCLC) [49]. Our recent findings also showed that PIK3R1, PIK3CG, PIK3R2, GNAI3, KRAS, NRAS, NFKB1, HLA-DMA, and HLA-DMB played central roles in benzene hematotoxicity and immune response signaling pathways such as B/T cell receptor signaling pathway, acute myeloid leukemia, hematopoietic cell lineage and natural killer cell mediated cytotoxicity were most significantly associated with benzene exposure [11].

These data, coupled with human epidemiology evidence linking benzene with cancers and recent evidence indicating DNA methylation alterations as a hallmark of cancer, support the notion that benzene exposure may lead to cancer in part via inducing the alterations of DNA methylation.

Unlike genetic mutations, DNA methylation is an inherently reversible change, and therefore is of great interest as an active target of drug development [50-52]. Because DNA methylation is potentially modifiable through lifestyle and pharmacological interventions, if confirmed, our findings may open new paths for prevention against benzene-induced carcinogenesis.

Previous studies have reported that the aberrant hypermethylation of promoter CpG islands is linked to gene silencing and loss of tumour suppressor function. DNA methylation of cytosine residues in CpG dinucleotides leads to transcriptional silencing of associated genes. Promoters with methylated CpG units, which have their transcriptional activity reduced, may function as an alternative mechanism of repressing tumour suppressor genes. Further research is needed to precisely define the mechanisms leading to benzene-induced DNA methylation changes. Our findings should be interpreted with caution, as there were a few limitations to this study. Further studies in other cell types and human samples are required, as well as determining the impact of these methylation changes on gene expression.

In conclusion, DNA methylation expression profiles along with GO, KEGG pathway annotation analysis have highlighted potential gene-based biomarkers of benzene exposure.

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References

1. Lan Q, Zhang L, Li G, Vermeulen R, Weinberg RS, et al. (2004) Hematotoxicity in workers exposed to low levels of benzene. Science 306: 1774-1776.
2. Rodríguez-Paredes M, Esteller M (2011) A combined epigenetic therapy equals the efficacy of conventional chemotherapy in refractory advanced non-small cell lung cancer. Cancer Discov 1: 557-559.
3. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, et al. (2004) Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 103:1635-1640.
4. Kuang SQ, Tong WG, Yang H, Lin W, Lee MK, et al. (2008) Genome-wide identification of aberrantly methylated promoter associated CpG islands in acute lymphocytic leukemia. Leukemia 22: 1529-1538.
5. Feinberg AP, Vogelstein B (1983) Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature. 301: 89-92.
6. Hou L, Zhang X, Tarantini L, Nordio F, Bonzini M, et al. (2011) Ambient PM exposure and DNA methylation in tumor suppressor genes: a cross-sectional study. Part Fibre Toxicol 8: 25.
7. Baccarelli A, Wright R, Bollati V, Litonjua A, Zanobetti A, et al. (2010) Ischemic heart disease and stroke in relation to blood DNA methylation. Epidemiology 21: 819-828.
8. Pearce MS, McConnell JC, Potter C, Barrett LM, Parker L, et al. (2012) Global LINE-1 DNA methylation is associated with blood glycaemic and lipid profiles. Int J Epidemiol 41: 210-217.
9. Ji Z, Zhang L, Peng V, Ren X, McHale CM, et al. (2010) A comparison of the cytogenetic alterations and global DNA hypomethylation induced by the benzene metabolite, hydroquinone, with those induced by melphalan and etoposide. Leukemia 24: 986-991.
10. Bollati V, Baccarelli A, Hou L, Bonzini M, Fustinoni S, et al. (2007) Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res 67: 876-880.
11. Gao A, Yang J, Yang G, Niu P, Tian L (2014) Differential gene expression profiling analysis in workers occupationally exposed to benzene. Sci Total Environ 472: 872-879.

12. Bai W, Yang J, Yang G, Niu P, Tian L, et al. (2014) Long non-coding RNA NR_045623 and NR_028291 involved in benzene hematotoxicity in occupationally benzene-exposed workers. Exp Mol Pathol 96: 354-360.

13. Carugno M, Pesatori AC, Dioni L, Hoxha M, Bollati V, et al. (2012) Increased mitochondrial DNA copy number in occupations associated with low-dose benzene exposure. Environ Health Perspect 120: 210-215.

14. Sandoval J, Heyn H, Moran S, Serra-Musach J, Pujana MA, et al. (2011) Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics 6: 692-702.

15. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, et al. (2011) High density DNA methylation array with single CpG site resolution. Genomics 98: 288-295.

16. Clarke R, Ressom HW, Wang A, Xuan J, Liu MC, et al. (2008) The properties of high-dimensional data spaces: implications for exploring gene and protein expression data. Nat Rev Cancer 8: 37-49.

17. Wright GW, Simon RM (2003) A random variance model for detection of differential gene expression in small microarray experiments. Bioinformatics 19: 2448-2455.

18. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, et al. (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25: 25-29.

19. Dupuy D, Bertin N, Hidalgo CA, Venkatesan K, Tu D, et al. (2007) Genome-scale analysis of in vivo spatiotemporal promoter activity in Caenorhabditis elegans. Nat Biotechnol 25: 663-668.

20. Draghici S, Khatri P, Tarca AL, Amin K, Done A, et al. (2007) A systems biology approach for pathway level analysis. Genome Res 17: 1537-1545.

21. Kanehisa M, Goto S, Kawashima S, Okuno Y, Hattori M (2004) The KEGG resource for deciphering the genome. Nucleic Acids Res 32: D277-280.

22. Yi M, Horton JD, Cohen JC, Hobbs HH, Stephens RM (2006) WholePathwayScope: a comprehensive pathway-based analysis tool for high-throughput data. BMC Bioinformatics 7: 30.

23. Li C, Li H (2008) Network-constrained regularization and variable selection for analysis of genomic data. Bioinformatics 24: 1175-1182.

24. Jansen R, Greenbaum D, Gerstein M (2002) Relating whole-genome expression data with protein-protein interactions. Genome Res 12: 37-46.

25. Wei Z, Li H (2007) A Markov random field model for network-based analysis of genomic data. Bioinformatics 23: 1537-1544.

26. Spirin V, Mirny LA (2003) Protein complexes and functional modules in molecular networks. Proc Natl Acad Sci U S A 100: 12123-12128.

27. Zhang JD, Wiemann S (2009) KEGGgraph: a graph approach to KEGG PATHWAY in R and bioconductor. Bioinformatics 25: 1470-1471.

28. Shen J, Wang S, Zhang YJ, Wu HC, Kibriya MG, et al. (2013) Exploring genome-wide DNA methylation profiles altered in hepatocellular carcinoma using Infinium HumanMethylation 450 BeadChips. Epigenetics 8: 34-43.

29. Krausz C, Sandoval J, Sayols S, Chianese C, Giachini C, et al. (2012) Novel insights into DNA methylation features in spermatozoa: stability and peculiarities. PLoS One 7: e44479.

30. Deneberg S, Guardiola P, Lennartsson A, Qu Y, Gaidzik V, et al. (2011) Prognostic DNA methylation patterns in cytogenetically normal acute myeloid leukemia are predefined by stem cell chromatin marks. Blood 118: 5573-5582.

31. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, et al. (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41: 178-186.

32. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, et al. (2014) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466: 253-257.

33. Laird PW (2010) Principles and challenges of genomewide DNA methylation analysis. Nat Rev Genet 11: 191-203.

34. Baccarelli A, Bollati V (2009) Epigenetics and environmental chemicals. Curr Opin Pediatr 21: 243-251.

35. Li Y, Wu XR, Li XF, Suriguga, Yu CH, et al. (2013) Changes in DNA methylation of erythroid-specific genes in K562 cells exposed to phenol and hydroquinone. Toxicology 312: 108-114.

36. Smirnikhina SA, Voronina ES, Lavrov AV, Bochkov NP (2013) Dioxidine-induced changes in genome-wide DNA methylation in a culture of peripheral blood lymphocytes. Bull Exp Biol Med 155: 224-227.

37. Gao A, Zuo X, Liu Q, Lu X, Guo W, et al. (2010) Methylation of PARP-1 promoter involved in the regulation of benzene-induced decrease of PARP-1 mRNA expression. Toxicol Lett 195: 114-118.

38. Gao A, Zuo X, Song S, Guo W, Tian L (2011) Epigenetic modification involved in benzene-induced apoptosis through regulating apoptosis-related genes expression. Cell Biol Int 35: 391-396.

39. Gu T, Gu HF, Hilding A, Sjöholm LK, Ostenson CG, et al. (2013) Increased DNA methylation levels of the insulin-like growth factor binding protein 1 gene are associated with type 2 diabetes in Swedish men. Clin Epigenetics 5: 21.

40. Wang XS, Zhang Y, He YH, Ma PP, Fan LJ, et al. (2013) Aberrant promoter methylation of the CD4 gene in peripheral blood cells of mastitic dairy cows. Genet Mol Res 12: 6228-6239.

41. Watkins DJ, Wellenius GA, Butler RA, Bartell SM, Fletcher T, et al. (2014) Associations between serum perfluoroalkyl acids and LINE-1 DNA methylation. Environ Int 63: 71-76.

42. Herman JG, Civin CI, Issa JP, Collector MI, Sharkis SJ, et al. (1997) Distinct patterns of inactivation of p15INK4B and p16INK4A characterize the major types of hematological malignancies. Cancer Res 57: 837-841.

43. Yin SN, Hayes RB, Linet MS, Li GL, Dosemeci M, et al. (1996) A cohort study of cancer among benzene-exposed workers in China: overall results. Am J Ind Med 29: 227-235.

44. Melki JR, Vincent PC, Clark SJ (1999) Concurrent DNA hypermethylation of multiple genes in acute myeloid leukemia. Cancer Res 59: 3730-3740.

45. Guo SX, Taki T, Ohnishi H, Piao HY, Tabuchi K, et al. (2000) Hypermethylation of p16 and p15 genes and RB protein expression in acute leukemia. Leuk Res 24: 39-46.

46. Seow WJ, Pesatori AC, Dimont E, Farmer PB, Albetti B, et al. (2012) Urinary benzene biomarkers and DNA methylation in Bulgarian petrochemical workers: study findings and comparison of linear and beta regression models. PLoS One 7: e50471.

47. Xing C, Chen Q, Li G, Zhang L, Zheng M, et al. (2013) Microsomal epoxide hydrolase (EPHX1) polymorphisms are associated with aberrant promoter methylation of ERCC3 and hematotoxicity in benzene-exposed workers. Environ Mol Mutagen 54: 397-405.

48. Greiner J, Ringhoffer M, Simikopinko O, Szmaragowska A, Huebsch S, et al. (2000) Simultaneous expression of different immunogenic antigens in acute myeloid leukemia. Exp Hematol 28: 1413-1422.

49. Hagihara A, Miyamoto K, Furuta J, Hiraoka N, Wakazono K, et al. (2004) Identification of 27 5' CpG islands aberrantly methylated and 13 genes silenced in human pancreatic cancers. Oncogene 23: 8705-8710.

50. Chen H, Suzuki M, Nakamura Y, Ohira M, Ando S, et al. (2006) Aberrant methylation of RASGRF2 and RASSF1A in human non-small cell lung cancer. Oncol Rep 15: 1281-1285.

51. Esteller M (2003) Profiling aberrant DNA methylation in hematologic neoplasms: a view from the tip of the iceberg. Clin Immunol 109: 80-88.

52. Rodríguez-Paredes M, Esteller M (2011) Cancer epigenetics reaches mainstream oncology. Nat Med 17: 330-339.

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