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Association of second hand smoke exposures with DNA methylation in bladder carcinomas

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Abstract

Background—The association between secondhand smoke (SHS) exposure and bladder cancer is inconclusive. Epigenetic alterations in bladder tumors have been linked to primary cigarette smoking and could add to the biological plausibility of an association between SHS exposure and bladder cancer.

Hypothesis—SHS exposure is associated with DNA methylation in bladder tumors.

Methods—Using an array-based approach, we profiled DNA methylation from never smoking cases of incident bladder cancer. Analyses examined associations between individual loci's methylation with SHS variables (exposure in adulthood, childhood, occupationally and total exposure). A canonical pathway analysis was used to find pathways significantly associated with each SHS exposure type.

Results—There is a trend towards increased methylation of numerous CpG loci with increasing exposure to adulthood, occupational and total SHS. Discrete associations between methylation extent of several CpG loci and SHS exposures demonstrated significantly increased methylation of these loci across all types of SHS exposure. CpGs with SHS-related methylation alterations were associated with genes in pathways involved in carcinogenesis, immune modulation and immune signaling.

Interpretation—Exposure to SHS in adulthood, childhood, occupationally and in total are each significantly associated with changes in DNA methylation of several CpG loci in bladder tumors, adding biological plausibility to SHS as a risk factor for bladder cancer.

Keywords

Bladder Cancer; Second Hand Smoking; DNA Methylation; Methylation Profiling; Array-based Methodologies

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Introduction

Bladder cancer is the ninth most incident form of cancer in the United States. Genetics, epigenetics, dietary factors, occupational exposures, environmental and lifestyle factors are all known to play important roles in bladder cancer initiation (1–5). The majority of bladder cancer cases are non-invasive and highly treatable, although these patients have great morbidity related to the need for intensive cystoscopic follow-up. Approximately 25% of cases present as muscle-invasive with high mortality rates (6–7). The biggest risk factor for bladder cancer is cigarette smoking, accounting for approximately 65% of disease risk in men and a smaller proportion in women (8). Tobacco smoke contains 2-naphthylamine, 4-aminobiphenyl and other arylamines amongst hundreds of other chemicals which experimental evidence has implicated as being bladder carcinogens (9–14). Environmental tobacco smoke or secondhand smoke (SHS) also contains these chemicals. Although the level of exposure may be less than in active smoking, the carcinogens present in SHS may make a contribution to bladder carcinogenesis, especially among people with higher susceptibility to tobacco toxicants (13). In fact, these toxicants have been detected in the urine of SHS-exposed individuals (15–16). However, only one case-control study among many epidemiological investigations found a positive association between exposure to SHS and risk of bladder cancer; and that risk was found to be present only in women (17–20).

Epigenetic mechanisms provide a heritable and stable form of gene expression control beyond the DNA sequence (21–22). A number of genes have been found to undergo epigenetic silencing through DNA methylation in bladder cancer (5, 23–24). Changes in the DNA methylation pattern of the promoter region of several cancer-related genes have also been associated with specific risk factors for bladder cancer, including smoking (5, 23–24).

Despite reported associations between DNA methylation and active smoking, the potential effect of SHS exposure on DNA methylation in bladder cancer remains unclear. Identifying associations between DNA methylation in tumors and SHS exposure would provide biological plausibility to the observed associations between SHS exposure and bladder cancer risk. Hence, we used an array-based DNA methylation profiling approach to examine the potential association between SHS exposure and DNA methylation in 1413 CpG loci, associated with 773 genes, in primary human bladder tumors from non-smoking individuals. Our aim was to conduct a pilot study examining these potential associations using a discovery-based platform in a small sample of bladder cancer cases. Identification of specific genes whose epigenetic alteration is associated specifically with SHS exposure may provide insight into important biological differences of these tumors, and suggest potential pathways for targeted chemotherapeutic or chemopreventative measures.

Materials and Methods

Subjects

A description of the study design appears in earlier reports (25–26). Briefly, bladder cancer cases were drawn from subjects enrolled in two stages of a non-consecutive, population-based case-control study of bladder cancer in New Hampshire, conducted from 1994–1998 and from 2001–2004. Cases of incident bladder cancer were identified from the state cancer registry and a standardized histopathologic review was conducted by a single study pathologist (A.R.S.) to verify the diagnosis and histopathology of the cases. Formalin-fixed, paraffin-embedded (FFPE) tumor tissue was obtained from the cases in the overall study. For the analyses presented here, the case group was restricted to non-smoking cases, with second hand smoke exposure (SHS) data, and excluded cases that were diagnosed as carcinoma in situ due to small numbers. We obtained promoter methylation data from all non-smoking cases in our sample giving us a final sample of 43 cases whose characteristics

are presented in Table 1. We noted no significant differences in the demographics or clinical characteristics of these non-smoking cases with SHS exposure data as compared to the remaining non-smoking cases without SHS exposure data. We also conducted additional sensitivity analyses on former and current smoking cases. For those analyses, the case group was restricted to cases having smoking status data and promoter methylation data and excluded cases that were diagnosed as carcinoma in situ due to small numbers. All procedures and study materials were approved by the appropriate Institutional Review Boards.

Personal Interview and Second Hand Smoking measurement

Consenting subjects underwent a detailed in-person interview, usually at their home, which assessed sociodemographic information, occupational history, detailed information about the use of tobacco products (such as history of cigarette smoking) and medical history prior to the reference or diagnosis date. Never smokers were defined as cases who had smoked less than 100 cigarettes over their lifetime. Among never smokers, SHS exposure was assessed through a questionnaire asking participants about the number of people who smoked around them everywhere they lived for at least 2 years duration since the age of 10 years and at the longest job they held for at least 6 months since the age of 16 years (25). Four types of second hand smoking exposure were assessed namely; childhood, adulthood, occupational, and total SHS exposure (cumulative exposure among childhood, adulthood and occupational). SHS exposures were calculated using the following measures: years spent living with ≥ 1 smoker in childhood (age ≤ 18), cumulative residential exposure in adulthood (the sum of the total number of smokers in each residence multiplied by the time spent in each residence over the person's lifetime), and the cumulative occupational SHS exposure (measured by the duration of time in years spent working with ≥ 1 smoker for the longest job that the subject held (as used in 25). Figure 1 shows the distribution of second hand smoke (SHS) exposure by each type of SHS exposure using boxplots.

DNA Methylation Analysis

DNA was extracted from the FFPE tumor samples as previously described (27). One μg of DNA was then subjected to sodium bisulfite modification using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) following the manufacturer's protocol. Methylation profiling was performed using the Illumina GoldenGate Methylation Bead Array at the UCSF Institute for Human Genetics Genomic Core Facility as previously described (28–29). All array data points were represented by fluorescent signals from both methylated (Cy5) and unmethylated (Cy3) alleles to create the average methylation (β) value derived from about 30 replicate methylation measurements, and which ranged from 0 (fully unmethylated) to 1 (fully methylated). The data was then assembled with BeadStudio methylation software from Illumina. Quality assurance and quality control (QA/QC) was performed and 8 poor performing loci (with >25% of samples having a detection p-value $>1.0\times10^{-5}$ at that locus) were removed. In addition, only autosomal CpG loci were considered, leaving 1413 CpG loci associated with 773 genes for analysis. In our previous work, we validated the results of the array using a bisulfite pyrosequencing, and observed strong correlation between the results of the array and this gold-standard method (30).

Statistical Methods

We calculated spearman correlations between each type of SHS exposure; spearman ranks were used to account for the potential nonparametric and nonlinear correlations between each type of SHS exposure. To identify specific loci whose differential methylation was associated with SHS exposure, we fit individual generalized linear models between the methylation beta value for each locus and the exposure. The beta distribution of average beta values was accounted for with a quasi-binomial logit link with an estimated scale parameter

constraining the mean between 0 and 1, as previously described (31). We accounted for multiple comparisons using false discovery rate estimation to calculate Q-values through the qvalue package in R (32). We also conducted individualized generalized linear models between the methylation beta value for each locus and former and current smoking status. To identify the cellular pathways represented by the genes associated with the differentially methylated loci, we conducted a canonical pathways analysis using the Ingenuity Pathway Analysis (Ingenuity Systems, 33). CpG loci that were found to be significantly associated with either adulthood, childhood, occupational or total second hand smoking exposure in the locus-by-locus analysis at the $Q<0.05$ level were considered for pathways analysis where all autosomal genes on the GoldenGate array were used as the referent gene set. The significance of gene-locus enrichment within canonical pathways was measured using a Fisher's exact test at the $p<0.05$ level. Data were analyzed by use of R, version 2.10.

Results

The characteristics of the cases used in these analyses, including SHS exposures are shown in Table 1, with the distribution of second hand smoke (SHS) exposure by each type of SHS exposure shown in Figure 1, using boxplots. In examining the correlation between the individual types of SHS exposure, we identified relatively modest correlations between adulthood, childhood, and occupational exposures ranging from −0.2 to 0.2 (Supplementary Table 1). As expected the correlation between each of the measures and the total exposure was greatest, as this is a cumulative measure of all exposure. Occupational exposures appeared to be the most correlated with total exposure, having a correlation coefficient of 0.8.

We examined whether each SHS exposure type was associated with specific methylation changes in the 1413 CpG loci in a locus-by-locus analysis. Figure 2 shows the −log(p-values) versus regression coefficients from locus-by-locus analysis of CpG methylation by type of exposure (Adulthood, Childhood, Occupational or Total) to SHS. A greater number of CpG loci show a significant, positive association between their methylation beta value and weighted measures of SHS exposure for all four types of exposure.

Those specific loci identified with the strongest associations (FDR $Q<0.05$) between methylation and SHS exposure are shown in Table 2. As expected from Figure 2, all of the loci reaching a $Q<0.05$ had increased methylation with increasing exposure to SHS. A total of 23 CpG loci were found to be significantly associated with adulthood exposure to SHS, while only 7 loci were found to be significantly associated with both childhood and total exposure to SHS. Only 3 CpG loci were found to be significantly associated with occupational exposure to SHS. Among CpGs with significant SHS associations, across exposure groups there were no CpGs in common.

To compare our results of epigenetic alterations associated with SHS exposure, we also looked at those specific loci identified with the strongest associations ($p<0.05$) between methylation and former and current smoking status in Supplemental Tables 2 and 3. After adjusting for multiple comparisons using FDR, only two loci were found to be marginally associated (at the $Q=0.06$ level) with current smoking status. Neither of those two overlapped with any of the specific loci that were identified with the strongest associations ($Q<0.05$) between methylation and any type of SHS exposure. However, two specific CpG loci (*EPHA1*_E46_R and *INHA*_P1144_R) that were found to be associated at the $p<0.05$ level with former smoking status were also associated with Childhood and Adulthood exposure to SHS respectively. Additionally, one specific CpG loci (*TGFA*_P642_R) that was found to be associated at the $p<0.05$ level with current smoking status was also associated with Adulthood exposure to SHS. In addition, members of the SFRP family were

found to be significantly associated (at the $p<0.05$ level) with former and current smoking status consistent with our previous work in another subset of bladder cancer cases from New Hampshire (27).

We performed a pathways analysis to determine which cellular pathways were over-represented amongst CpG loci identified in the locus-by-locus analysis as significantly ($Q<0.05$) associated with adulthood, childhood, occupational or total SHS exposure. For CpG loci significantly associated with adulthood SHS exposure we found that cancer-related, JAK/Stat, IL-4, and renin-angiotensin signaling pathways were significantly over-represented (Table 3). For CpG loci significantly associated with exposure to SHS in childhood we found that the ERK5, glioma, and T-Cell receptor signaling pathways were significantly over-represented (Table 3). Among CpGs significantly associated with occupational SHS exposure, the hepatic fibrosis/hepatic stellate cell activation pathway was significantly over-represented (Table 3). Lastly, for CpG loci significantly associated with total SHS exposure, the growth hormone, erythropoietin, JAK/Stat, IL-3, IL-4, and the CTLA4 signaling in cytotoxic T lymphocytes pathways were over-represented (Table 3).

Discussion

This study aimed to investigate the association between SHS exposure and DNA methylation of a large number of CpG loci bladder cancer cases who had never smoked. We tested the hypothesis that epigenetic changes in bladder tumors are associated with SHS exposure. Our finding of specific CpG loci methylation being associated with either adulthood, childhood, occupational or total SHS exposure, provides considerable additional evidence for the biological plausibility for the association between SHS exposure and bladder cancer risk, and may aid in explaining the incidence of bladder cancer amongst non-primary smoking individuals. CpG loci with statistically significant associations between their methylation status and SHS exposures demonstrated exclusively greater extents of methylation suggesting that SHS exposure may induce and/or select for specific hypermethylation events in bladder cancer.

Previous work has shown associations between methylation of the promoter region of several cancer-related genes in bladder tumors and primary tobacco smoking, particularly identifying associations between current and recent former smoking and hypermethylation of the *CDKN2A* and *RASSF1A* genes (23). A propensity for hypermethylation of a panel of tumor suppressor genes was also significantly more pronounced in current smokers (23–24). Our results suggest that SHS exposure may also lead to hypermethylation of key tumor suppressor genes. Interestingly, we did not identify those loci whose hypermethylation was previously found to be associated with primary smoking exposures, including *CDKN2A*, *RASSF1A*, MDM2, or the *SFRP* family (23, 27, 34). This was confirmed when we ran individualized generalized linear models between the methylation beta value for each locus and former and current smoking status, where we found no overlap with any of the specific loci that were identified with the strongest associations (FDR $Q<0.05$) between methylation and any type of SHS exposure. We did find that two specific CpG loci that were found to be associated at the $p<0.05$ level with former smoking status were also associated with Childhood and Adulthood exposure to SHS respectively, and that one specific CpG loci that was found to be associated at the $p<0.05$ level with current smoking status was also associated with Adulthood exposure to SHS. This may suggest that the types of exposure encountered by the bladders of SHS-exposed individuals are different than those of primary smoking individuals, and thus contribute different selective pressures resulting in differential methylation. In addition, the fact that the genes whose CpGs were significantly associated with either adulthood, childhood or occupational SHS exposure were all distinct from one another, suggest that the specific type or timing of SHS exposure is important epigenetically.

Indeed, there may be variability in the way the different SHS exposures were experienced; this could lead to stochastic epigenetic changes due to the less consistent way individuals may experience these individual types of SHS exposures. These SHS exposures are themselves likely to be less intense and less consistent than those experienced by primary smokers. We also checked the correlation between our SHS exposures and key bladder cancer risk factors (namely age, gender, tumor grade and stage) and found that these covariates largely are not associated with the change in methylation observed between exposed and unexposed individuals (reflected by low correlation coefficients).

A number of CpG loci associated with genes in various cellular receptor kinase families were found to have significantly greater methylation associated with individual types of SHS exposure. The insulin-like growth factor-I receptor (*IGF1R*) gene and the insulin-like growth factor-II receptor (*IGF2R*) gene have opposite roles in oncogenesis with *IGF1R* acting as a mediator in tumor cell growth (as shown in pancreatic cancer) by inhibiting tumor apoptosis, while *IGF2R* acts as a tumor suppressor decreasing cellular proliferation and increasing apoptosis (35–36). *IGF2R* has been found to be hypermethylated in ovarian and breast tumors showing that both *IGF1R* and *IGF2R* could potentially be good targets for alterations by the components of SHS exposure (36). Other receptor kinases found to be hypermethylated with exposure to SHS were the fms-related tyrosine kinase 1 (*FLT1*), the EPH receptor B2 (*EPHB2*) and the ephrin-A1 (*EFNA1*) gene (37–39). All three have been shown to have aberrant methylation in tumors, with *FLT1* showing CpG island methylator phenotype (CIMP)-associated DNA hypermethylation in colorectal tumors, *EPHB2* showing altered methylation of its promoter regulatory sequences in colorectal tumors, and *EFNA1* being hypermethylated in leukemia cells (37–39). Also of particular interest was the hypermethylation of O6-methylguanine-DNA methyltransferase (*MGMT*) associated with adulthood SHS exposure. *MGMT* is involved in DNA repair and has been found to be aberrantly hypermethylated in tumors such as breast cancer (40). Its methylation state in glioma serves as a clinically useful marker of susceptibility to the chemotherapy temozolamide (41). Finally, the connective tissue growth factor (*CTGF*) was found to be hypermethylated after exposure to both occupational and total SHS. *CTGF* is a secreted protein belonging to the CCN family shown to be hypermethylated in ovarian cancer; it may therefore be a factor in the carcinogenesis of ovarian cancer (42).

Of the pathways found to be significantly enriched amongst genes associated with any type of exposure to second hand smoking in our canonical pathways analysis, the JAK/Stat, IL-3 IL-4, CTLA4, T-cell receptor, and erythropoietin signaling pathways were all found to be significantly associated with adulthood and total exposure to SHS. The pathways are of interest, both in that they have been linked to tumorigenesis, and are critical regulators of the immune response (43–48). The role of the immune system in bladder cancer is of great interest as patients with localized disease are often treated with Bacillus Calmette-Guérin therapy, which is thought to illicit a local inflammatory response against the bladder tumor. Epigenetic alteration of key regulators of these immune responses may help to explain differential response to this treatment.

The extracellular-signal-regulated kinase 5 (*ERK5*) and the renin-angiotensin signaling pathways were also enriched amongst genes whose methylation status was associated with SHS exposure (49–50). This suggests that these exposures may be influencing vascular remodeling and angiogenic pathways with may be critical in sustaining or enhancing tumor growth (49–50).

Strengths of this study included the population-based nature of the study, as well as the use of the Illumina GoldenGate Methylation Bead Array for methylation profiling. Limitations of this study include the small sample size and the use of a questionnaire to ascertain second

hand smoking exposure of study participants, as their responses may have been subject to recall bias. Future studies with larger sample sizes and adequately detailed SHS data are warranted.

In summary, this study demonstrates that passive smoking, like active smoking is associated with DNA methylation across numerous CpG loci in bladder cancer, but appears to be targeting different genes than those that have been associated with active smoking. The novelty of these results lies in the use of array-based methodologies to examine methylation of a large number of CpG loci in relation to SHS to help further clarify the potential association and biological plausibility of the risk of bladder cancer and SHS exposure. These findings indicate that SHS exposure has effects on methylation levels of genes and in turn affect important biological pathways which may have impacts on overall bladder cancer risk. However, due to our small sample size, we recognize the necessity to replicate and extend these results in a larger sample in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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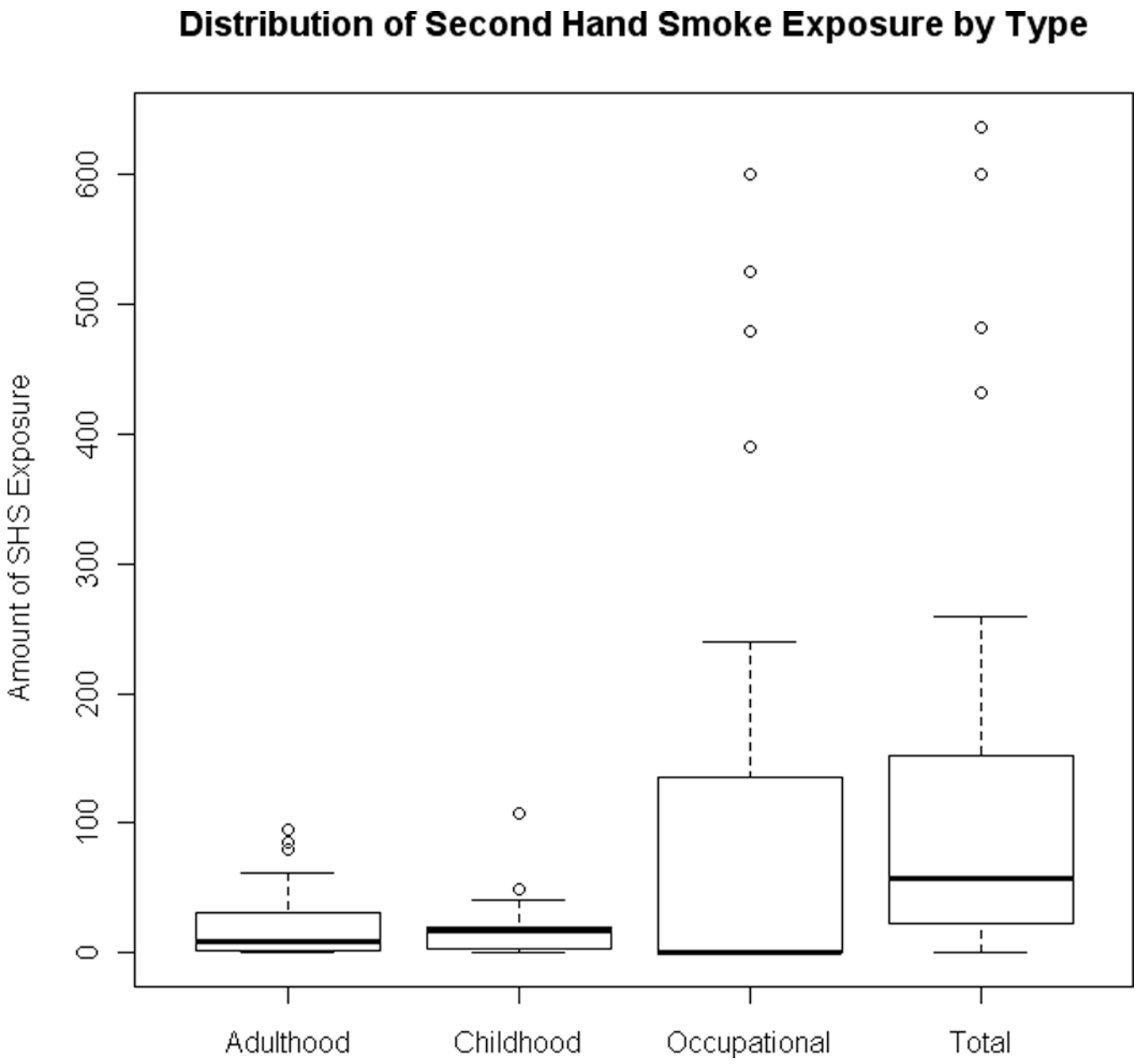

Figure 1.
The distribution of second hand smoke (SHS) exposure by each type of SHS exposure is shown using boxplots. A boxplot is a graphical technique used to depict the range of a variable, with the 5th and the 95th percentiles of the variable being shown as the whiskers of the box and with the lower quartile (25th percentile) and the upper quartile (75th percentile) being shown as the edges of the box. The median or 50th percentile is shown by the horizontal line through the box. A boxplot can not only show the range of the data but also gives us a sense of potential outlying observations of a certain variable.

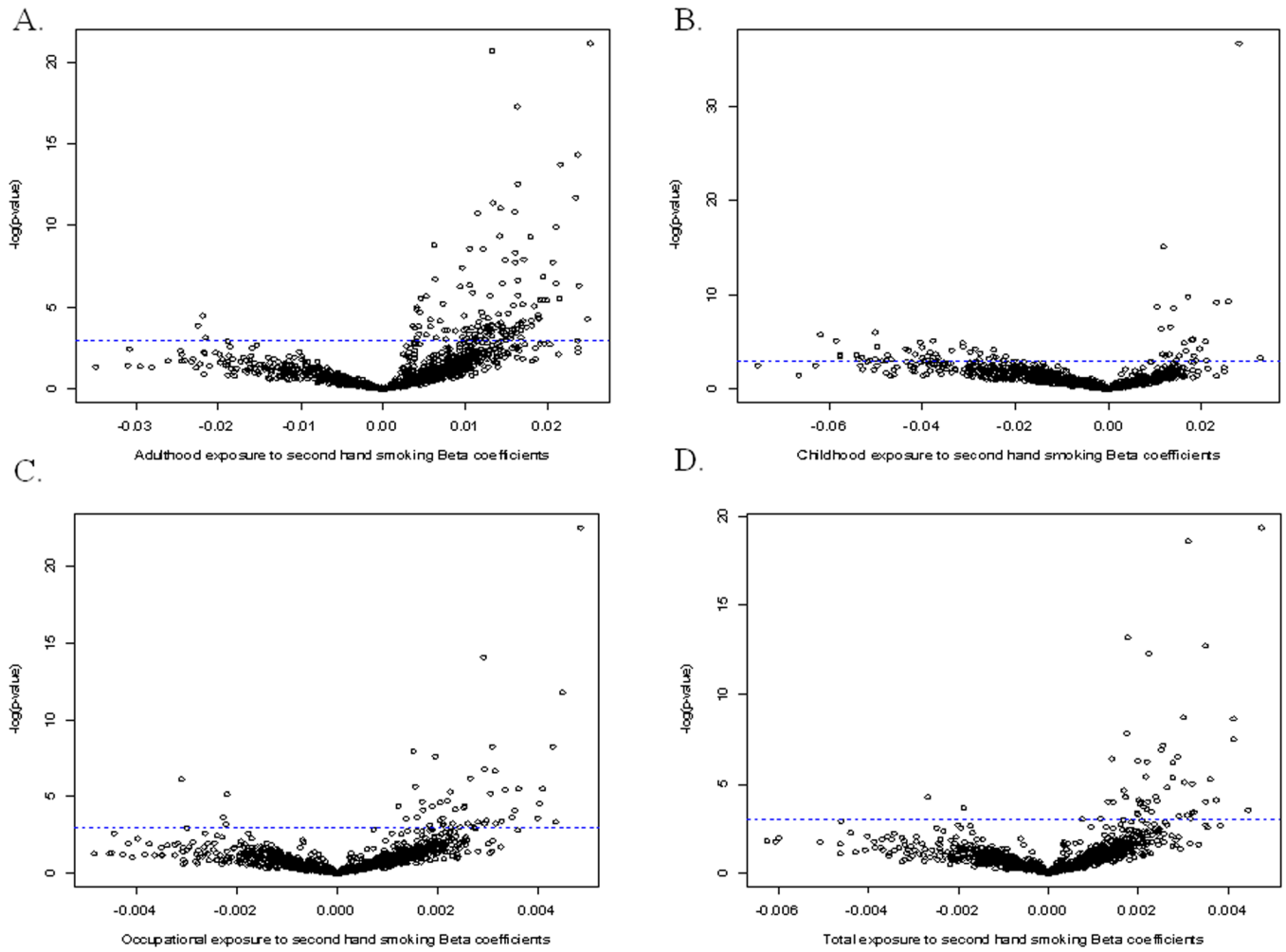

Figure 2.

Volcano plots volcano plot of the −log (p-values) plotted against the quasi binomial regression coefficients from the locus-by-locus analysis of 1413 CpG methylation values by (A) adulthood second hand smoking, (B) childhood second hand smoking, (C) occupational second hand smoking, and (D) total second hand smoking. The horizontal blue dotted line intercepts the y-axis to illustrate significance above the line (before correction for multiple comparisons).

Table 1

Selected characteristics of bladder cancer cases with second hand smoking status data

Characteristics	Bladder Cancer Cases (N=43)
Gender, n (%)	
Female	14 (32.6%)
Male	29 (67.4%)
Age, mean (SD)	63 (11.5)
Tumor Stage*	
Non-invasive	30 (75.0%)
Invasive	10 (25.0%)
Second hand smoking variables, mean(SD)†	
Adulthood Passive ††	19.5 (24.5)
Childhood Passive ±	18.6 (19.5)
Occupational Passive //	81.9 (151.1)
Total Passive: Childhood, Adulthood, Occupational	119.9 (153.8)

* not available on 3 subjects

† Weighted by years of exposure

†† Sum of the years spent at each adult residence multiplied by the number of smokers in each residence

± Sum of the years spent at each childhood residence (up to age 18 years) multiplied by the number of smokers in each residence

// Sum of the years spent at the longest job multiplied by the number of Smokers

Table 2

Significant loci (at the Q-value<0.05 level) by second hand smoking exposure type:

Genes	Specific CpG loci	Q-value	Type of second hand smoking exposure
FLT1	FLT1_E444_F	0.036	Total
PTPN6	PTPN6_E171_R	0.036	Total
CTSH	CTSH_P238_F	0.001	Total
EPHB2	EPHB2_P165_R	0.001	Total
CD9	CD9_E14_R	0.001	Total
JAK2	JAK2_P772_R	0.000	Total
CTGF	CTGF_E156_F	0.000	Total
FLT1	FLT1_E444_F	0.004	Occupational
JAK2	JAK2_P772_R	0.001	Occupational
CTGF	CTGF_E156_F	0.000	Occupational
SHB	SHB_P473_R	0.037	Childhood
SMARCA3	SMARCA3_P109_R	0.037	Childhood
EPHA1	EPHA1_E46_R	0.028	Childhood
VAMP8	VAMP8_P114_F	0.028	Childhood
LIF	LIF_E208_F	0.027	Childhood
IGF2R	IGF2R_P396_R	0.000	Childhood
RRAS	RRAS_P100_R	0.000	Childhood
BCL3	BCL3_P1038_R	0.029	Adulthood
TGFA	TGFA_P642_R	0.022	Adulthood
TYK2	TYK2_P494_F	0.022	Adulthood
ITPR3	ITPR3_P1112_F	0.021	Adulthood
YES1	YES1_P216_F	0.021	Adulthood
LIG4	LIG4_P194_F	0.015	Adulthood
FHIT	FHIT_P93_R	0.012	Adulthood
IL12A	IL12A_E287_R	0.012	Adulthood
PPARG	PPARG_E178_R	0.011	Adulthood
PKD2	PKD2_P336_R	0.007	Adulthood
RET	RET_seq_54_S260_F	0.007	Adulthood
MGMT	MGMT_P272_R	0.005	Adulthood
NR2F6	NR2F6_E375_R	0.002	Adulthood
TYRO3	TYRO3_P501_F	0.002	Adulthood
IGF1R	IGF1R_E186_R	0.002	Adulthood
INHA	INHA_P1144_R	0.002	Adulthood
EPHB2	EPHB2_P165_R	0.001	Adulthood
CDH3	CDH3_E100_R	0.001	Adulthood
DDR1	DDR1_E23_R	0.000	Adulthood
PTPN6	PTPN6_E171_R	0.000	Adulthood
CTSH	CTSH_P238_F	0.000	Adulthood
CD9	CD9_E14_R	0.000	Adulthood

Genes	Specific CpG loci	Q-value	Type of second hand smoking exposure
EFNA1	EFNA1_P591_R	0.000	Adulthood

Table 3

Pathways over-represented amongst loci whose methylation was significantly associated with second hand smoking exposure

Pathway	Type of second hand smoke exposure	p-value	Genes
Non-Small Cell Lung Cancer Signaling	Adulthood	0.004	*FHIT, NRAS, ITPR3, TGFA*
Thyroid Cancer Signaling	Adulthood	0.014	*PPARG, NRAS, RET*
JAK/Stat Signaling	Adulthood	0.020	*PTPN6, NRAS, TYK2*
IL-4 Signaling	Adulthood	0.023	*PTPN6, NRAS, TYK2*
Renin-Angiotensin Signaling	Adulthood	0.040	*PTPN6, NRAS, ITPR3*
ERK5 Signaling	Childhood	0.036	*LIF, RRAS*
Glioma Signaling	Childhood	0.048	*RRAS, IGF2R*
T Cell Receptor Signaling	Childhood	0.048	*SHB, RRAS*
Hepatic Fibrosis / Hepatic Stellate Cell Activation	Occupational	0.027	*CTGF, FLT1*
JAK/Stat Signaling	Total	0.033	*PTPN6, JAK2*
Growth Hormone Signaling	Total	0.034	*PTPN6, JAK2*
Erythropoietin Signaling	Total	0.035	*PTPN6, JAK2*
IL-4 Signaling	Total	0.036	*PTPN6, JAK2*
IL-3 Signaling	Total	0.037	*PTPN6, JAK2*
CTLA4 Signaling in Cytotoxic T Lymphocytes	Total	0.046	*PTPN6, JAK2*