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RESEARCH

Association of BLCA-4 Hypomethylation in Blood Leukocyte DNA and the Risk of Bladder Cancer in a Chinese Population

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Abstract Global DNA hypomethylation has been associated with increased risk for cancers of the colorectum, bladder, breast, head and neck, and testicular germ cells. The aim of this study was to examine whether global hypomethylation measured at BLCA-4 repeat regions through bisulfite pyrosequencing in blood leukocyte DNA is associated with the risk of bladder cancer(BC). A total of 312 bladder cancer patients and 361 healthy control subjects were included in Chongqing, China. Global methylation in blood leukocyte DNA was estimated by analyzing BLCA-4 repeats using bisulfite-polymerase chain reaction (PCR) and pyrosequencing. The median methylation level in BC cases (percentage of 5-methylcytosine (5 mC)=75.7 %) was significantly lower than that in controls (79.7 % 5 mC) (P=0.002, Wilcoxon rank-sum test). The odds ratios (ORs) of BC for individuals in the third, second, and first (lowest) quartiles of BLCA-4 methylation were 1.2 (95 % confidence interval (CI) 0.8–1.9), 1.6 (95 % CI 1.1–2.3), and 2.7 (95 % CI 1.5–3.8) (P for trend <0.001), respectively, compared to individuals in the fourth (highest) quartile. A 2.1-fold (95 % CI 1.5–2.8) increased risk of BC was observed among individuals with BLCA-4 methylation below the median compared to individuals with higher (>median) BLCA-4 methylation. Our results demonstrate for the first time that individuals with global hypomethylation measured in BLCA-4 repeats in blood leukocyte DNA have an increased risk for BC. Our data provide the evidence that BLCA-4 hypomethylation may be a useful biomarker for poor prognosis of patients with BC.

Keywords BLCA-4 · Hypomethylation · Bladder cancer · Blood leukocyte

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Introduction

Bladder cancer is the ninth most common form of cancer in the world, with men having three to four times the risk of women [1]. Smoking remains the greatest risk factor for developing the disease, with smokers having a fourfold increase in risk compared with nonsmokers [2, 3]. Other risk factors include occupational chemical exposures, spinal cord injuries, age, and diet [4]. Epigenetic changes in methylation patterns are increasingly implicated in cancer development [5, 6]. Global DNA hypomethylation seems to occur in early neoplasia and has been regarded as an important component of cancer development [5, 7]. Compared with normal tissue counterparts, lower global DNA methylation was observed in tumor tissues in a broad panel of cancers [5]. Animal studies have shown that experimentally induced hypomethylation can lead to cancer development at multiple sites [6, 8, 9], indicating the causal role of global DNA hypomethylation in cancer development. In addition, lower levels of global methylation were reported in leukocyte DNA from subjects with colorectal adenoma, the precursor of colorectal cancer [10, 11]. Further, in healthy populations, global hypomethylation in blood leukocyte DNA has been associated with exposure to environmental risk factors for carcinogenesis, such as benzene, tobacco smoke, persistent

organic pollutants, and perfluorooctane sulfonate [12–16]. Several case-control studies have shown that global hypomethylation measured in blood DNA was associated with an increased risk for cancers of the colorectum, bladder, breast, head and neck, and testicular germ cells [10, 17–21], suggesting that global DNA hypomethylation is a potential biomarker of cancer susceptibility. Global DNA methylation level derives from the overall 5-methylcytosine (5 mC) dinucleotide CpG sites in the human genome, about 55 % of which consists of repetitive elements [22]. BLCA-4, identified in the proteomic surveys as one of the six nuclear matrix proteins (NMPs) that are bladder cancer specific [23], has yielded the highest sensitivity and specificity thus far in a series of studies [24]. Reduced levels of global DNA methylation, assessed in peripheral blood, have been associated with bladder cancer risk in European and American populations. In view of heavy methylation in normal tissue, high representation throughout the genome, and close correlation with genomic DNA methylation content, we suppose BLCA-4 methylation status may be used as a surrogate marker for estimating the genomic DNA methylation level. In the present case-control study based on 312 HCC cases and 361controls from a Chinese population, we sought to determine the association between BLCA-4 hypomethylation and the risk of BC.

Materials and Methods

Subjects

This is a hospital-based case-control study. A total of 312 newly diagnosed BC patients were recruited from Southwest Hospital Affilated to Third Military Medical University, Chongqing, China from February 2003 to October 2009. All cases did not receive chemotherapy or radiation therapy before blood sample collection. Final diagnoses were pathologically confirmed from the specimens obtained by surgery. As a control, blood samples of 361 cancer free subjects were randomly selected from the individuals who attended for the physical examination at the same hospitals during the period when the case patients were recruited. The controls were frequency-matched to the cases by age (±5 years), and sex status. For both case and control subjects, those with other cancers, diabetes, autoimmune disorders, or other major systemic diseases were excluded. Information on age, sex, habits of alcohol drinking and cigarette smoking, and BC family history was obtained using structured questionnaire through in-person interviews. An ever-drinker was defined as a person who reported drinking alcoholic beverages at least once per week for at least 6 months. An ever-smoker was defined as a smoker of at least five cigarettes per day for at least 6 months. Tumor characteristics including tumor size, and tumor stage (the 1973 World Health Organization criteria [25]) were made on the basis of medical records. Written informed consent was obtained from each participant. The study protocol was approved by the ethics review committee of the Institutional Review Board of the two participant hospitals.

DNA Extraction and Bisulfite Modification

Genomic DNA of blood leukocytes was extracted using the QIAamp DNA Blood Mini kit(Qiagen, Shanghai, China). Extracted DNA was treated with sodium bisulfite using the EZ DNA Methylation kit (Zymo, CA, USA) according to the manufacturer's protocol.

BLCA-4 Polymerase Chain Reaction (PCR) and Pyrosequencing

Briefly, PCR was carried out in a 50 μl reaction volume containing 25 μl of GoTaq Green Master mix (Promega, WI, USA), 1 pmol of forward primer (TTTTG AGTTAGGTGTGGGATATA), 1 pmol of biotinylated reverse primer (biotin-AAAATCAAA AAATTCCC TTTC), and 50 ng of bisulphite-treated DNA. PCR conditions were 40 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. PCR product was bound to streptavidin sepharose beads(Amersham Biosciences, Uppsala, Sweden), and then was purified, washed, denatured, and washed again. Then, 0.3 μmol/L pyrosequencing primer (AGTTAGGTGTGGGATATAGT) was annealed to the purified PCR product. Pyrosequencing actions were performed in the PSQ HS 96 Pyrosequencing System. The non-CpG cytosine residues in BLCA-4 repetitive sequences, which were rarely methylated, were used as built-in controls to verify bisulfite conversion, and complete conversion of cytosine at non-CpG sites ensured successful bisulfite conversion. The percentage of 5 mC at each of three CpG dinucleotide positions in BLCA-4 repetitive sequences was measured. The degree of BLCA-4 methylation was expressed as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (percentage of 5 mC). To validate PCR pyrosequencing assay, each CpG dinucleotide position was assayed in duplicate and their averages were used in final analysis. The within-sample coefficient of variation was 0.68 %, which suggests good reliability in the measurement.

Statistical Analysis

Differences between BC cases and controls in age, sex, alcohol drinking, cigarette smoking, and BC family history were evaluated using the $\chi 2$-test. The nonparametric comparisons of median BLCA-4 methylation levels between BC

cases and controls were evaluated by Wilcoxon rank-sum test. The association of BLCA-4 methylation with BC risk was estimated using the odds ratios (ORs) and 95 % confidence intervals (CIs) from multivariate logistic regression analyses, with adjustment for age, sex, alcohol drinking, cigarette smoking, and BC family history. Statistical analyses were conducted using the Stata 10.1(Stata Corp., College Station, TX). All tests were two-sided and a P value of ≤0.05 was considered statistically significant.

Results

The general characteristics of the BC cases and controls are shown in Table 1. No significant differences between BC cases and controls were found in the distribution of age, sex, alcohol drinking, and BC family history. Compared with controls, more cases had cigarette smoking habits (P=0.02).

Table 1 Characteristics of BC and control subjects

Variable	Number of subjects[a]		P[b]
	BC cases (n=312)	Controls (n=361)	
Age			
≤50 years	146 (46.8 %)	167 (46.3 %)	0.86
>50 years	166 (53.2 %)	194 (53.7 %)	
Sex			
Female	70 (22.4 %)	83 (23.0 %)	0.93
Male	242 (77.6 %)	278 (77.0 %)	
Alcohol drinking			
Never	184 (59.0 %)	201 (55.7 %)	0.39
Ever	128 (41.0 %)	160 (44.3 %)	
Cigarette smoking			
Never	153 (49.0 %)	209 (57.9 %)	0.02
Ever	159 (51.0 %)	152 (42.1 %)	
BC family history[c]			
No	294 (94.2 %)	339 (93.9 %)	0.87
Yes	18 (5.8 %)	22 (6.1 %)	
Tumor size			
≤3cm	152 (48.7 %)		
>3cm	160 (51.3 %)		
Grade			
G1	65 (20.8 %)		
G2-G3	247 (79.2 %)		
T stage			
Ta-T1	201 (64.4 %)		
T2-T4	111 (36.6)		

[a] Data are expressed as n (%).[b] χ^2 -test; [c] Family history of BC in first-degree relatives

Figure 1 depicts the distribution of the BLCA-4 methylation levels in BC cases and controls as box plots of the data. The median methylation level (75.7 % 5 mC) in cases was significantly lower than that (79.7 % 5 mC) in controls (P=0.002). Logistic regression analysis showed that the levels of BLCA-4 methylation were inversely associated with the risk of BC (Table 2). The odds ratios (ORs) of BC for individuals in the third, second, and first (lowest) quartiles of BLCA-4 methylation were 1.2(95 % confidence interval (CI) 0.8–1.9), 1.6(95 % CI 1.1–2.3), and 2.7 (95 % CI 1.5–3.8) (P for trend <0.001), respectively, compared to individuals in the fourth (highest) quartile. A 2.1-fold (95 % CI 1.5–2.8) increased risk of BC was observed among individuals with BLCA-4 methylation below the median, compared to individuals with higher (>median) BLCA-4 methylation.

Stratification by BC risk factors including age (≤50 years, >50 years), sex (female, male), alcohol drinking (never, ever), cigarette smoking (never, ever), and BC family history (no, yes), produced comparable results, regardless of quartile or median BLCA-4 methylation evaluated (data not shown). Additional analyses showed that the interactions between BLCA-4 methylation and these BC risk factors in relation to BC risk were not statistically significant (data not shown).

No significant associations were observed of age, alcohol drinking, cigarette smoking, or BC family history with BLCA-4 methylation, based on control subjects. However, males (79.6 % 5 mC) had higher BLCA-4 methylation than females (78.3 % 5 mC, P=0.03) among controls. No significant correlationwas found between BLCA-4 methylation and tumor size, tumor grade or tumor stage (data not shown).

Discussion

Global hypomethylation in blood leukocyte DNA has been reported to correlate with increased risk for several types of

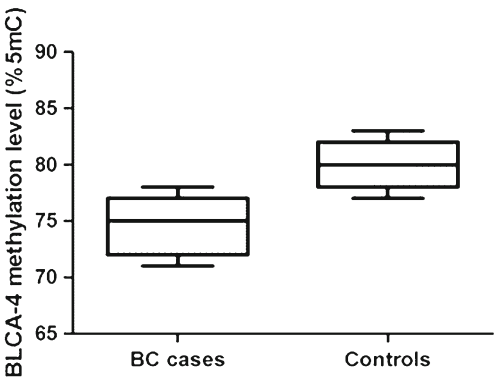

Fig. 1 Comparison of BLCA-4 methylation levels measured in blood leukocyte DNA in BC cases and controls (P=0.002)

Table 2 Association of BLCA-4 methylation with risk of BC

BLCA-4 (% 5 mC)	Number of subjects[a]		OR(95 % CI)[b]	P[b]
	BC cases (n=312)	Controls (n=361)		
Quatile[c]				
Q4	51 (16.3 %)	90 (24.9 %)	1[d]	
Q3	59 (18.9 %)	88 (24.4 %)	1.2 (0.8–1.9)	0.72
Q2	69 (22.1 %)	92 (25.5 %)	1.6 (1.1–2.3)	0.24
Q1	133 (42.6 %)	91 (25.2 %)	2.7 (1.5–3.8)	<0.001
Median[c]				
High	108 (34.6 %)	176 (48.8 %)	1[d]	
Low	204 (65.4 %)	185 (51.2 %)	2.1 (1.5–2.8)	<0.001

[a]Data are expressed as n (%); [b]Adjusted for age, sex, alcohol drinking, cigarette smoking, and BC family history; [c]The quartiles and the median of BLCA-4 measures were based on values among control subjects; [d]Reference. P for trend <0.001 for the ORs of BC from the fourth, third, second to first quartiles of BLCA-4 methylation

cancer [10, 17–21]. In the present study, we demonstrated for the first time that individuals with global hypomethylation measured in BLCA-4 repeats in leukocyte DNA had an increased BC risk. Taken together, these findings support the idea that there exist common epigenetic basis for the pathogenesis of these different cancers. DNA methylation is a reversible epigenetic mechanism and DNA methylation patterns can be changed in response to exposure to exogenous and endogenous factors [26, 27]. Global hypomethylation in leukocyte DNA has been reported to be associated with exposure to risk factors for carcinogenesis [12–16], suggesting that leukocyte DNA hypomethylation may reflect cumulative effects from carcinogenic exposures. On the other hand, global methylation change has been indicated to be partly under genetic control [28, 29], suggesting that DNA hypomethylation in leukocyte DNA may also reflect transgenerational risks for common human diseases including cancers [28]. Thus, global methylation levels in leukocyte DNA could provide a useful biomarker of susceptibility to certain cancer types.

Global DNA hypomethylation has been proposed to contribute to activation of oncogenes and genomic instability in cancer tissues, thereby causing aberrant activation of a wide spectrum of genes, formation of abnormal chromosomal structures and increased mutation rates that convey various growth advantages [6, 30]. On the other hand, oncogenes can be activated by the derepression of endogenous repeats, in addition to genetic and epigenetic modifications [31]. Loss of imprinting (LOI), which is involved in the development of tumors, is also associated with DNA hypomethylation [32]. Furthermore, it has also been suggested that the activation of normally silenced genes by promoter DNA hypomethylation is involved in tumorigenesis [33]. BCLA-4 is a nuclear transcription factor present in bladder tumors and adjacent benign areas of the bladder, but not in benign urothelium. BLCA-4 is one of six such factors which are promising tumor markers in bladder cancer detection. This protein is tested by an ELISA on voided urine. Preliminary studies indicate a sensitivity of 89 %–96 % with a specificity of 100 % for bladder cancer [34, 35]. Global DNA hypomethylation has been demonstrated to lead to the transcriptional activation of BLCA-4 promoters, which can alter the transcriptome both in primary bladder tumors and in their premalignant urothelium counterparts [36]. These findings suggested that hypomethylation of BLCA-4 may play a role not only in cancer but also in cancer predisposition.

Our study had the advantages of being based on a relatively large sample size, careful matching of controls to BC cases by age, and sex status, and pyrosequencing-based quantitative analysis which produced individual measures of methylation at three CpG dinucleotide positions, thus more accurately reflecting DNA methylation in the region. Several limitations in the present study should be noted. Firstly, this was a hospital-based case-control study, which may result in selection bias of participants. Moreover, given that DNA methylation in cancer patients was measured after cancer diagnosis and DNA hypomethylation could be detected in sera as well as BC cells in peripheral blood of BC cases, blood DNA hypomethylation in cancer patients we observed may partly represent the methylation level of DNA derived from BC cells. As degree of hypomethylation in tumor cells and number of BC cells in circulating peripheral blood progress according to increased tumor stage, further analyses of BLCA-4 methylation in relation to BC risk were repeated using cases only with early stage of tumor (Stage a-1), which showed no major differences in risk estimates from the results based on the total number of cases (data not shown). In this context, the potentially-confounding effect of contaminating DNA from BC cells on the association between BLCA-4 hypomethylation and BC risk observed in the present study would be minimal. However, further prospective studies using BC-free subjects are needed to verify the risk-effect of global hypomethylation in blood DNA on BC development. Secondly, information on diet was not available here, which may also influence methylation levels in genomic DNA [37, 38].

In conclusion, global hypomethylation measured in BLCA-4 repeats in blood leukocyte DNA shows an increased risk for BC. Furthermore, BLCA-4 hypomethylation in patients with BC may be associated with a poor prognosis and may thus serve as a useful molecular biomarker for predicting prognosis. Larger studies are needed to confirm these findings as well as to investigate the essentially unknown mechanisms of BLCA-4 hypomethylation.

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