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Genomic DNA hypomethylation and risk of renal cell carcinoma: A case-control study

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Abstract

Purpose—Genomic DNA hypomethylation is a hallmark of most cancer genomes, promoting genomic instability and cell transformation. In the present study, we sought to determine whether global DNA methylation in peripheral blood is associated with risk of renal cell carcinoma (RCC).

Experimental Design—A retrospective case control study consisting of 889 RCC cases and an equal number of age, gender, and ethnicity-matched controls was applied. Global DNA methylation was measured as 5-mC% content. Logistic regression was used to estimate odds ratio (OR) and 95% confidence interval (CI) for the association between DNA methylation level and the risk of RCC.

Results—The median 5-mC% was significantly lower in cases than healthy controls ($p<0.001$). In multivariate logistic regression analysis, individuals in the lowest tertile (T1) of 5-mC% had higher risk of RCC with OR of 1.40 (95%CI 1.06–1.84), compared to individuals in the highest tertile (T3) ($P_{for\ trend}=0.02$). When stratified by RCC risk factors, associations between hypomethylation and increased RCC risk appeared to be stronger among males (OR=1.61, $P_{for\ trend}=0.01$), younger age (OR=1.47, $P_{for\ trend}=0.03$), never smokers (OR=1.55, $P_{for\ trend}=0.02$), family history of other cancer (OR=1.64, $P_{for\ trend}=1.22E-03$) and late stage (OR=2.06,

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Conflict of Interest

All authors have no conflicts of interest to disclose.

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$P_{for\ trend}$=4.98E-04). Additionally, we observed significant interaction between gender and 5-mC% in elevating RCC risk ($P_{for\ interaction}$=0.03).

Conclusions—Our findings suggest an association between global DNA hypomethylation and RCC risk. To establish global DNA hypomethylation as a risk factor for RCC, future prospective studies are warranted. This study may provide further understanding of the etiology of RCC tumorigenesis.

Keywords

RCC; kidney cancer; cancer risk; 5-mC%; DNA methylation

INTRODUCTION

Kidney cancer represents 3.8% of all new cases in the United States (US) (1). Adult kidney cancers are mainly adenocarcinomas, also known as a renal cell carcinoma (RCC). RCC accounts for more than 90% of adult kidney carcinomas (2). The incidence of RCC has been steadily rising by 2–4% each year and is now the 8th leading cancer type in the US. Despite improved diagnosis, about 20–30% of all RCC patients have developed metastases at the time of diagnosis and an additional 30–50% progress to metastatic disease during follow-up (3). The overall 5-year survival rate of RCC patients is in the range of 50–60%; however, the long-term survival diminishes in patients with distant metastasis (4). Previous studies reported that having a first degree relative with kidney cancer is associated with 2 to 3-fold increased risk (5). In the US, RCC incidence differs among racial and ethnic populations: African Americans have both higher incidence and mortality rates than other races/ethnicities (6). Cigarette smoking, hypertension and obesity are established risk factors associated with RCC development (7). Dietary intake of vegetables and fruits has been inversely associated with RCC. Greater intake of red meat and milk products has been associated with increased RCC risk, although not consistently (8).

Lifestyle and environmental factors associated with RCC carcinogenesis likewise affect epigenetic statuses, and thus epigenetic mechanisms may mediate environmental influences on gene expression and cancer development. DNA methylation is one of the most studied epigenetic modifications in mammals. The covalent addition of a methyl group occurs mostly in cytosine within CpG dinucleotides, which are concentrated in large clusters called CpG islands. It is known that inactivation of certain tumor-suppressor genes occurs as a consequence of hypermethylation in the promoter regions, and genomic instability resulting from global hypomethylation promotes cell transformation. In addition, global DNA hypomethylation contributes less frequently to activation of silenced oncogenes (9). In normal cells, pericentromeric heterochromatin is highly methylated; satellite sequences and repetitive genomic sequences (such as LINE, SINE, IAP and Alu elements) are silenced, thereby ensuring genomic integrity and stability. However, in a variety of tumors, this mechanism is disrupted and loss of DNA methylation occurs. As a consequence, there is a chance of undesired mitotic recombination, and transposable elements can be reactivated and integrated at random sites in the genome, leading to mutagenesis and genomic instability. Also, loss of DNA methylation may activate latent viral sequences in the genome, which can contribute to tumor progression (9).

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Recently, global hypomethylation in peripheral blood DNA has been associated with increased risks of various cancers including head and neck (10), gastric (11), liver (12), bladder (13–15), colorectal (16–18) and breast cancer (19–22). Although alterations in DNA methylation from peripheral blood may not necessarily represent epigenetic changes in the tumor, the noninvasiveness of acquiring blood samples and the likelihood of tumor heterogeneity requiring multiple assessments of tumor tissues make the surrogate approach attractive. Global hypomethylation in leukocytes may reflect overall genomic instability of an individual, which may predispose to cancer development (19). To date, there was only one report in RCC in which higher levels of LINE-1 methylation were positively associated with RCC risk, particularly among current smokers (23). In the present study, we used a large case control study to evaluate the association of global DNA methylation levels of blood DNA with the risk of RCC, and investigate if any modifiable risk factors involved in the development of RCC could modify this association.

MATERIAL AND METHODS

Study Population

This is an ongoing case control study of RCC that has been recruiting RCC patients from the University of Texas MD Anderson Cancer Center in Houston, Texas, since 2002. The study design was described previously (24). Briefly, all recruited cases were individuals with newly diagnosed (within 1 year of diagnosis), histologically confirmed, and previously untreated RCC. Healthy control subjects without a history of cancer were identified and recruited with the use of random digit dialing (RDD) method. The control subjects were frequency matched to the cases according to gender, ethnicity and age (±5 years). The overall response rate for RDD screening was 51% and, among those who agreed to participate, the response rate was 88%. The response rate for the eligible cases was 87%. The study was approved by the MD Anderson Institutional Review Board. The blood was collected before patients received treatment at MD Anderson. All participants provided written informed consent before participating in the study. Immediately after each interview, a 40_ml blood sample was drawn into heparinized tubes for lymphocyte isolation and DNA extraction.

Data Collection

Epidemiological data were collected by MD Anderson interviewers in a 45-min structured in-person interview. Data included information regarding history of hypertension (yes/no), smoking status and pack-years of smoking, physical activity and usual weight, weight at age 20 and 40 years, and family history of cancer was recorded.

Smoking status and pack-years of smoking were defined as such. An individual who had never smoked or had smoked <100 cigarettes in his or her lifetime was defined as a never smoker. An individual who had smoked at least 100 cigarettes in his or her lifetime but had quit at least 12 months before diagnosis (for cases) or before the interview (for controls) was classified as a former smoker. Current smokers were those who were currently smoking or quit <12 months before diagnosis (for cases) or before the interview (for controls). The number of pack-years was calculated as the average number of cigarettes smoked per day

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divided by 20 cigarettes and then multiplied by smoking years. Weight at diagnosis (for cases) or recruitment (for control subjects) was recorded. Body mass index (BMI; kg/m2) was calculated through self-reported usual height and weight. BMI was categorized according to the standard classifications of the World Health Organization (normal = <25 kg/m2; overweight = 25–29.9 kg/m2; obese = ≥30 kg/m2). Participants also reported the average frequency they spent on five broad groups of physical activities in the year before the interview. A metabolic equivalent (MET) value was assigned to each activity group and categorized into low (MET<27 per week), medium (MET 27–44.9 per week) and intensive (MET≥45 per week) (25).

Family history data included cancer history for all first-degree relatives (biological parents, siblings, and offspring). Specifically, the information collected included whether the relative ever had cancer (yes or no), the type of cancer (site), age at diagnosis, current age or age of death, vital status (dead or alive), smoking status (yes or no), years smoked, number of cigarettes smoked, and whether the relative ever had high blood pressure (yes or no).

Global DNA methylation analysis

Genomic DNA was isolated from peripheral blood using the QIAamp DNA blood maxi kit (QIAGEN, Valencia, CA). Quantitative and qualitative DNA analysis was performed using Nanodrop. The global DNA methylation was measured by the MethylFlash™ Methylated DNA Quantification Kit (Epigentek Inc., Farmingdale, NY USA) according to the manufacturer's instructions. The capture antibody in this kit binds to 5-methylcytosine, thus measuring total DNA methylation level as a percentage of total DNA present in the sample. A standard curve was run as were the positive and negative controls. Inter-assay coefficient of variation (CV) was <15%.

Briefly, 100 ng of DNA (20_ng/μl) was bound to the plate at 37°C for 90_min. The methylated fraction of DNA was detected using capture and detection antibodies and then the relative optical density (OD) units were quantified by reading the absorbance in a FLUOstar Optima microplate reader (BMG Labtech). The amount of methylated DNA was proportional to the OD measured. The absolute amount of methylated DNA was quantified from a standard curve, and the slope of the standard curve was used to calculate the percentage of methylated DNA (5-mC%) in the sample.

Statistical analysis

Continuous variables and amounts of global methylation were categorized into dichotomous or tertile variables based on the distribution among controls. Chi-square test was used to compare characteristics between cases and controls for categorical variables such as gender, smoking status, ethnicity, hypertension, family history of cancer, and categorized continuous variables (BMI, BMI at age 20, BMI at age 40, and physical activity). Student’s t-test was used for the differences in means of continuous variables between cancer cases and healthy controls (i.e. age, pack-year smoking, BMI, and 5-mC%). The Wilcoxon rank-sum test was used as a nonparametric comparison of median methylation levels in cases versus controls. To identify potential predictors of methylation levels and/or factors that may modify the association between methylation levels and RCC risk, univariate associations between DNA

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methylation and each lifestyle or demographic factor were assessed among cases and all participants (cases and controls). To assess the associations between factors that may be related to global DNA methylation, we measured standardized β-coefficients among controls and all participants. A positive estimate (β-coefficient) of the correlation between the risk factor and the global methylation level reflects an increasing 5-mC% response to the risk factor; a negative estimate reflects a diminishing (hypomethylation) 5-mC% response to the risk factor. Additionally, global methylation levels (5-mc%) were categorized into three groups using the tertile cutoff points among all the control subjects for each category of the various covariates.

To evaluate the association between the global DNA methylation levels (5-mC%) and RCC risk, conditional logistic regression model adjusted for gender, age and ethnicity was initially used to estimate odds ratio (OR) and 95% confidence intervals (CI). The 5mC% content was defined using the tertile cutoff points among all the control subjects and the same cutoff points were used for all the subgroup analyses. The highest tertile (T3) of methylation (≥5.25) was used as the reference. We subsequently performed a multivariable-adjusted stratified analysis of risk according to known and suspected RCC risk factors (Supplementary Table 1). The final model included gender (male or female), age (categorical variable, ≤60 and >60), ethnicity (Caucasian, Hispanic and African-American), smoking status (never, former and current), BMI (<25, 25–30 and ≥30), hypertension (yes or no), physical activity (low, medium and intensive) and family cancer history (no cancer history, kidney cancer or other cancer history). Potential interactions were evaluated using multiplicative interaction by including the cross-product term of 5-mC% variable and the risk factor variables in the logistic regression model. Significance of the interactions was determined using the Wald statistic for the cross-product term. Analyses were repeated for population subgroups based on the results stratified by possible confounders. All statistical analyses were conducted using STATA version 9.0 (Stata corporation, College Station, TX). All tests were two-sided and a *P* value of ≤0.05 was considered statistically significant.

RESULTS

The characteristics of the cases and controls are shown in the Table 1. A total of 1,778 participants were enrolled in this study, including 889 RCC cases and an equal number of age, gender, and ethnicity-matched controls. The overall population was largely Caucasian (84% in cases and controls) with an average age of 59 years old, over two-thirds were males and ~50% were never smokers in cases and controls. Hypertension, BMI, physical activity and family history of cancer were significantly different between cases and controls; unexpectedly, we did not observe significant difference by smoking status, perhaps due to the possible presence of selection bias in our clinic-based study population. Most of the cases were of clinical stage I (47.58%) and clear cell RCC (73%). Compared with the controls, RCC cases were more likely to have a history of hypertension (p=5.81E-15), higher BMI (p= 0.01), low physical activity (p= 1.48E-20), and a family history of cancer (other than kidney cancer) (p= 5.42E-07). Overall, the median 5-mC% methylation level in cases was 3.64, significantly lower than the median level of 3.97 in controls (p<0.001 Wilcoxon rank-sum test).

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We analyzed the effect of demographics, clinic-pathological and lifestyle factors on global DNA methylation level in controls and among all participants (cases and controls). We used standardized β-coefficients to measure the estimates of the correlation. Global methylation levels were not associated with any of the demographics and lifestyle factors examined among controls Table 2 and Supplementary Table 1). Among all participants, on the other hand, showed significant correlations between BMI and physical activity and global DNA methylation were observed. Specifically, we saw a significant 21% decrease in the methylation level for subjects with higher BMI at age 40 (p=0.03) and significant 20% increase in the methylation level for subjects with intensive physical activity (p=0.03) (Table 2). Association with genomic methylation levels did not vary by tumor characteristics, such as histological cell type and clinical stage.

As we observed differences in the distribution of 5-mC% methylation level between cases and controls, we performed logistic regression to elucidate the association of global DNA methylation level and risk of RCC, adjusted for known and suspected risk factors and cofounders (Table 3). In this model, the 5mC% content was defined using the tertile cutoff points among control subjects and the same cutoff points were used for all the subgroup analyses. The highest tertile (T3) of methylation (≥5.25) was used as the reference. Results from the gender-, age- and ethnicity adjusted conditional logistic regression analyses suggest that individuals with low 5-mC% levels exhibit significantly increased risk of RCC (OR=1.46, 95%CI 1.15–1.84, p for trend 1.49E-03)(Table 3). In multivariate analysis adjusted for gender, age, ethnicity, smoking status, BMI, hypertension, physical activity and family cancer history, individuals in the lowest tertile (T1) and middle tertile of 5-mC% had higher risks of RCC with ORs of 1.40 (95%CI 1.06–1.84) and 1.30 (95%CI 0.98–1.72) compared to individuals in the highest tertile (T3) of methylation (p for trend=0.02) (Table 3).

In the stratified analyses, associations between hypomethylation and increased RCC risk appeared to be stronger among males (OR=1.61, 95%CI 1.14–2.28, p for trend 0.01), younger age at diagnosis (OR=1.47, 95%CI 1.04–2.08, p for trend=0.03), never smokers (OR=1.55, 95%CI 1.08–2.23, p for trend 0.02), family history of other cancer (OR=1.64, 95%CI 1.21–2.20, p for trend=1.22E-03) and late stage (OR=2.06, 95%CI 1.36–3.12, p for trend=4.98E-04) Results also suggested significant interaction between gender and global DNA methylation in elevating RCC risk (p for interaction=0.03) (Table 4).

DISCUSSION

This case-control study showed that the median amount of global DNA methylation measured as 5-mC% content in leukocyte DNA was significant lower in cases than in controls and were independently associated with increased RCC risk in a dose-dependent manner. We found a significant 1.4-fold increased risk of RCC among subjects in the lowest tertile of methylation, compared with subjects in the highest tertile. Our results are consistent with previous studies in patients with head and neck cancer (10), bladder cancer (14, 15, 26), testicular cancer (27), breast cancer (28), gastric cancer (11) and hepatocellular carcinoma (12), where the authors reported lower methylation levels of repetitive elements were associated with increased risk of cancer. Global DNA hypomethylation of leukocyte

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DNA, measured as 5-mC levels, has been associated with increased cancer risks in several studies, including bladder (13), colorectal adenoma (16, 17) and breast cancer (19). These studies are consistent with the fact that hypomethylation is promoting genomic instability and cell transformation. In contrast, a previous RCC case control study by Liao *et al.* measured LINE-1 methylation levels among 328 RCC cases and 654 controls from the central and eastern European renal cancer study that was conducted in seven centers in Eastern and Central Europe and found that the median LINE-1 methylation levels in leukocyte DNA were higher in RCC cases (81.97%) than in controls (81.67%, p=0.003, Wilcoxon test). Compared with individuals in the lowest LINE-1 methylation quartile, individuals in the highest quartile were associated with a 2-fold increased risk of RCC (p for trend= 0.004) (23). The discrepancy between our current study and that study could be due to different population and different techniques. Only a third of genomic DNA methylation is estimated to occur in repetitive elements (29) and its methylation level is not equivalent to global DNA methylation content (19). On the other hand, DNA methylation measured as 5-mC content have been used in several epidemiological studies (13, 19, 30) as an alternate marker for global DNA methylation in entire genome. Though the number of methylation screening methods has expanded broadly and different approaches have been used to measure genomic DNA methylation, no single technique fulfills all criteria for generating unambiguous data on methylation (31). The percentage of 5-methylcytosine (5-mC%) using monoclonal antibodies against 5-mC allows the estimation of genomic DNA methylation levels and, compared with the measure of repetitive genomic regions such as LINE-1 or Alu, is a powerful tool which provides a direct measurement and permit the study of global DNA methylation contents in a highly quantitative way. No correlation between 5-mC% and LINE-1 were found in a previous study that found difference in methylation level between breast cancer cases and controls using the former but not latter method, suggesting differential sensitivity in the two methods to detect global DNA methylation (19). Furthermore, Phokaew et al. reported that LINE-1 methylation levels of white blood cells was highly variable depending on where the targeted sequence are located, suggesting that the targeted locus for LINE-1 methylation measurement should be cautiously selected (32). Several other methods are available to measure global DNA methylation but have not been widely used in epidemiological studies due to limitations in throughput, accuracy, and cost. Further investigation is needed to validate various quantification methods for global DNA methylation and to determine whether global DNA hypomethylation is a marker of cancer risk.

Epigenetic changes, particularly DNA methylation, are susceptible to change and may explain how certain environmental factors increase the risk of cancer. Several studies have reported significant associations between global DNA hypomethylation and the exposure to cancer risk factors (33–36), suggesting that DNA hypomethylation could be the result of carcinogenic exposures. Previous epidemiologic studies have investigated the association between demographic, environmental and lifestyle risk factors with global DNA methylation in leukocytes. The age-dependent decrease of global DNA methylation has been reported previously (37–39). Many studies have found that global DNA methylation was higher in males than in females (10, 14, 37–39). The association between race/ethnicity and global DNA methylation was investigated in a few studies with conflicting results (10, 26, 43).

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Zhang, et al reported higher levels of methylation in Caucasians than Hispanic and non-Hispanic black groups (43), but opposite findings were reported in another study (10). The association between global DNA methylation in leukocytes and smoking habits (10, 13, 39–42), alcohol drinking, BMI and physical activity have also been investigated but the results were not consistent (12, 39, 43). Though, despite all of these studies, inconsistent levels arise due to the challenges of interpreting results from different assays and from different sources of DNA. There are still no epidemiologic studies examining whether changes in white blood cells methylation over time are associated with changes in disease endpoints. Large prospective studies will be needed to understand whether DNA methylation levels measured in blood samples represent a useful biomarker. To date, how global DNA hypomethylation increases cancer risk is less well understood. Evidence suggests that DNA hypomethylation functions in direct or indirect control of transcription and in destabilizing chromosomal integrity (29). The genome of the transformed cell undergoes simultaneously a global genomic hypomethylation and a dense hypermethylation of the CpG islands associated with gene regulatory regions (30). Three mechanisms may explain how DNA hypomethylation contributes to carcinogenesis: chromosomal instability, reactivation of transposable elements, and loss of imprinting. Low levels of methylation might favor mitotic recombination leading to loss of heterozygosity, karyotypically detectable rearrangements, and illegitimate expression. Additionally, extensive demethylation in centromeric sequences is common in human tumors and may play a role in aneuploidy (30). Global DNA hypomethylation may also cause activation of a wide spectrum of genes including oncogenes that contribute to proliferation, differentiation and cancer transformation (9).

The data of this case-control study suggest that the impact of global DNA methylation in increasing RCC risk may be limited to males, younger individuals, never smokers, late clinical stage and those with another family cancer history besides kidney cancer. Even though the exact mechanism for these differential associations remains unclear, our results suggest that the role of DNA hypomethylation in contributing to cancer risk may be more substantial for individuals without traditional RCC risk factors. Although gender did not affect the level of DNA methylation, it did modify the association between global DNA methylation and risk of RCC. In a stratified analysis by gender, the risk was higher in males than in females. However, unlike previous reports (13, 14), we observed higher methylation levels in females. Kidney cancer is most frequently diagnosed among people aged 55–64 years old, in our study we found a more pronounced increased risk conferred by hypomethylation among individuals younger than 60 years old, suggesting that age may modify the effect of global hypomethylation on RCC risk.

Adult lifestyle factors such as smoking were reported to be associated with lower levels of genomic DNA methylation in individuals with head and neck tumors (26). In this study, stratified analyses showed that the association between DNA methylation and RCC risk was only evident in never smokers but not in former and current smokers. This observation is consistent with previous studies in bladder (13, 15) and breast cancer (19) where the authors reported stronger association of reduced LINE-1 methylation with increased risks of cancers among never smokers. It is established that tobacco smoking is a risk factor for renal cell cancer. The cellular modifications resulting from exposure to the chemicals present in the cigarette smoke have been widely investigated and include DNA adducts, gene mutations,

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micronuclei, chromosome aberrations, sister chromatin exchanges and DNA strands breaks (44). However, given that smoking presumed to cause about 30% of kidney cancers in men and approximately 25% in women in the United States (1), our findings of the association between global hypomethylation and higher risk of RCC among never smokers, may add understanding to the biological mechanism of non-smoking related RCC carcinogenesis, though this conclusion may not be accurate and should be take carefully due to the lack of association between tobacco smoking and RCC in our data. Further research in this area is necessary to elucidate these associations.

Finally, global DNA methylation levels were not related with progression of the disease, we did not find changes in methylation levels between early and late stage RCC cases. We observed a higher risk of RCC associate with DNA hypomethylation in late stage cases, suggesting an independent effect of hypomethylation increasing RCC risk in this particular group; however given that the methylation levels were measured in DNA from blood cells and not in the tumor, and the sample collection time was right after cancer diagnosis, care must be taken interpreting these results.

Our study has several strengths including a relatively large sample size, careful matching of controls to RCC cases by age, gender, and ethnicity, and the use of 5-mC% content as a surrogate marker for global DNA methylation in the entire genome. Moreover, this is the first study to take into account suspected RCC risk factors, such as physical activity, as well as traditional risk factors in reference to the association between global DNA methylation and RCC risk. As this is a retrospective, cross-sectional case-control study, we cannot establish the temporal relationship and causality between DNA hypomethylation and the risk of developing RCC. Aberrant methylation is a hallmark of the carcinogenic process. Although we only included newly diagnosed RCC patients (within 1 year of diagnosis) for which blood samples were drawn at recruitment and before treatment to minimize the potential impact of disease and treatment on global DNA methylation, we cannot rule out the possibility of “reverse causation.” To establish global DNA hypomethylation as a risk factor for RCC, future prospective studies are warranted. Second, one of the methodological issues with our work is the utilization of genomic DNA isolated from whole peripheral blood, since differences in DNA methylation levels may result from changes in the relative proportions of blood cell subtypes present in each sample or due to different environmental exposures (45). Given the lack of cell purification in our study and possible variation in immune cell populations in individual samples, care should be taken in interpreting our results and when comparing data across different molecular epidemiologic studies. Additional research is needed to understand the association between levels of global DNA methylation in different blood cell subtypes. Although the methodology of sample collection (e.g. time from blood draw to processing of sample; freezing and storage conditions) might affect the integrity of 5-mC, the same protocols for blood collection, processing, storage and DNA extraction were applied for all specimens analyzed in this study.

In summary, we report that low genomic DNA methylation in peripheral blood was significantly associated with increased risk of RCC; further studies in large prospective cohort studies are warranted to confirm our findings. More systemic approach is needed to investigate the influence of various RCC risk factors, such as BMI and physical activity, on

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DNA methylation and RCC development. So far, the results of this study add to the growing body of evidence that hypomethylation of epigenome may be associated with increased cancer risk. Assessing the level of global DNA methylation in peripheral blood may provide additional phenotypic marker for RCC risk estimation, which merits further study in other cancer types. Changes in the epigenome may offer clues to unveil the etiologic mechanism of RCC tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin. 2015; 65:5–29. [PubMed: 25559415]
2. Purdue MP, Moore LE, Merino MJ, Boffetta P, Colt JS, Schwartz KL, et al. An investigation of risk factors for renal cell carcinoma by histologic subtype in two case-control studies. Int J Cancer. 2013; 132:2640–7. [PubMed: 23150424]
3. Cohen HT, McGovern FJ. Renal-cell carcinoma. N Engl J Med. 2005; 353:2477–90. [PubMed: 16339096]
4. Lam JS, Klatte T, Kim HL, Patard JJ, Breda A, Zisman A, et al. Prognostic factors and selection for clinical studies of patients with kidney cancer. Crit Rev Oncol Hematol. 2008; 65:235–62. [PubMed: 17931881]
5. Noordzij MA, Mickisch GH. The genetic make-up of renal cell tumors. Urol Res. 2004; 32:251–4. [PubMed: 15497212]
6. Stafford HS, Saltzstein SL, Shimasaki S, Sanders C, Downs TM, Sadler GR. Racial/ethnic and gender disparities in renal cell carcinoma incidence and survival. J Urol. 2008; 179:1704–8. [PubMed: 18343443]
7. Dhote R, Thiounn N, Debre B, Vidal-Trecan G. Risk factors for adult renal cell carcinoma. Urol Clin North Am. 2004; 31:237–47. [PubMed: 15123404]
8. Chow WH, Devesa SS. Contemporary epidemiology of renal cell cancer. Cancer J. 2008; 14:288–301. [PubMed: 18836333]
9. Kulis M, Esteller M. DNA methylation and cancer. Adv Genet. 2010; 70:27–56. [PubMed: 20920744]
10. Hsiung DT, Marsit CJ, Houseman EA, Eddy K, Furniss CS, McClean MD, et al. Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev. 2007; 16:108–14. [PubMed: 17220338]
11. Hou L, Wang H, Sartori S, Gawron A, Lissowska J, Bollati V, et al. Blood leukocyte DNA hypomethylation and gastric cancer risk in a high-risk Polish population. Int J Cancer. 2010; 127:1866–74. [PubMed: 20099281]
12. Di JZ, Han XD, Gu WY, Wang Y, Zheng Q, Zhang P, et al. Association of hypomethylation of LINE-1 repetitive element in blood leukocyte DNA with an increased risk of hepatocellular carcinoma. J Zhejiang Univ Sci B. 2011; 12:805–11. [PubMed: 21960343]
13. Moore LE, Pfeiffer RM, Poscablo C, Real FX, Kogevinas M, Silverman D, et al. Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case-control study. Lancet Oncol. 2008; 9:359–66. [PubMed: 18339581]

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14. Wilhelm CS, Kelsey KT, Butler R, Plaza S, Gagne L, Zens MS, et al. Implications of LINE1 methylation for bladder cancer risk in women. Clin Cancer Res. 2010; 16:1682–9. [PubMed: 20179218]
15. Cash HL, Tao L, Yuan JM, Marsit CJ, Houseman EA, Xiang YB, et al. LINE-1 hypomethylation is associated with bladder cancer risk among nonsmoking Chinese. Int J Cancer. 2012; 130:1151–9. [PubMed: 21445976]
16. Lim U, Flood A, Choi SW, Albanes D, Cross AJ, Schatzkin A, et al. Genomic methylation of leukocyte DNA in relation to colorectal adenoma among asymptomatic women. Gastroenterology. 2008; 134:47–55. [PubMed: 18166347]
17. Pufulete M, Al-Ghnaniem R, Leather AJ, Appleby P, Gout S, Terry C, et al. Folate status, genomic DNA hypomethylation, and risk of colorectal adenoma and cancer: a case control study. Gastroenterology. 2003; 124:1240–8. [PubMed: 12730865]
18. Huang WY, Su LJ, Hayes RB, Moore LE, Katki HA, Berndt SI, et al. Prospective study of genomic hypomethylation of leukocyte DNA and colorectal cancer risk. Cancer Epidemiol Biomarkers Prev. 2012; 21:2014–21. [PubMed: 23001241]
19. Choi JY, James SR, Link PA, McCann SE, Hong CC, Davis W, et al. Association between global DNA hypomethylation in leukocytes and risk of breast cancer. Carcinogenesis. 2009; 30:1889–97. [PubMed: 19584139]
20. Jackson K, Yu MC, Arakawa K, Fiala E, Youn B, Fiegl H, et al. DNA hypomethylation is prevalent even in low-grade breast cancers. Cancer Biol Ther. 2004; 3:1225–31. [PubMed: 15539937]
21. Soares J, Pinto AE, Cunha CV, Andre S, Barao I, Sousa JM, et al. Global DNA hypomethylation in breast carcinoma: correlation with prognostic factors and tumor progression. Cancer. 1999; 85:112–8. [PubMed: 9921982]
22. Esteller M, Fraga MF, Guo M, Garcia-Foncillas J, Hedenfalk I, Godwin AK, et al. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet. 2001; 10:3001–7. [PubMed: 11751682]
23. Liao LM, Brennan P, van Bemmel DM, Zaridze D, Matveev V, Janout V, et al. LINE-1 methylation levels in leukocyte DNA and risk of renal cell cancer. PLoS One. 2011; 6:e27361. [PubMed: 22076155]
24. Clague J, Lin J, Cassidy A, Matin S, Tannir NM, Tamboli P, et al. Family history and risk of renal cell carcinoma: results from a case-control study and systematic meta-analysis. Cancer Epidemiol Biomarkers Prev. 2009; 18:801–7. [PubMed: 19240244]
25. Ainsworth BE, Haskell WL, Whitt MC, Irwin ML, Swartz AM, Strath SJ, et al. Compendium of physical activities: an update of activity codes and MET intensities. Med Sci Sports Exerc. 2000; 32:S498–504. [PubMed: 10993420]
26. Terry MB, Ferris JS, Pilsner R, Flom JD, Tehranifar P, Santella RM, et al. Genomic DNA methylation among women in a multiethnic New York City birth cohort. Cancer Epidemiol Biomarkers Prev. 2008; 17:2306–10. [PubMed: 18768498]
27. Mirabello L, Savage SA, Korde L, Gadalla SM, Greene MH. LINE-1 methylation is inherited in familial testicular cancer kindreds. BMC Med Genet. 2010; 11:77. [PubMed: 20478068]
28. Cho YH, Yazici H, Wu HC, Terry MB, Gonzalez K, Qu M, et al. Aberrant promoter hypermethylation and genomic hypomethylation in tumor, adjacent normal tissues and blood from breast cancer patients. Anticancer Res. 2010; 30:2489–96. [PubMed: 20682973]
29. Ehrlich M. Cancer-linked DNA hypomethylation and its relationship to hypermethylation. Curr Top Microbiol Immunol. 2006; 310:251–74. [PubMed: 16909914]
30. Esteller M, Herman JG. Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. J Pathol. 2002; 196:1–7. [PubMed: 11748635]
31. Dahl C, Guldberg P. DNA methylation analysis techniques. Biogerontology. 2003; 4:233–50. [PubMed: 14501188]
32. Phokaew C, Kowudtitham S, Subbalekha K, Shuangshoti S, Mutirangura A. LINE-1 methylation patterns of different loci in normal and cancerous cells. Nucleic Acids Res. 2008; 36:5704–12. [PubMed: 18776216]

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33. Bollati V, Baccarelli A, Hou L, Bonzini M, Fustinoni S, Cavallo D, et al. Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res. 2007; 67:876–80. [PubMed: 17283117]
34. Rusiecki JA, Baccarelli A, Bollati V, Tarantini L, Moore LE, Bonefeld-Jorgensen EC. Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environ Health Perspect. 2008; 116:1547–52. [PubMed: 19057709]
35. Breton CV, Byun HM, Wenten M, Pan F, Yang A, Gilliland FD. Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. Am J Respir Crit Care Med. 2009; 180:462–7. [PubMed: 19498054]
36. Liu F, Killian JK, Yang M, Walker RL, Hong JA, Zhang M, et al. Epigenomic alterations and gene expression profiles in respiratory epithelia exposed to cigarette smoke condensate. Oncogene. 2010; 29:3650–64. [PubMed: 20440268]
37. Fuke C, Shimabukuro M, Petronis A, Sugimoto J, Oda T, Miura K, et al. Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study. Ann Hum Genet. 2004; 68:196–204. [PubMed: 15180700]
38. Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, et al. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mech Ageing Dev. 2009; 130:234–9. [PubMed: 19150625]
39. Zhu ZZ, Hou L, Bollati V, Tarantini L, Marinelli B, Cantone L, et al. Predictors of global methylation levels in blood DNA of healthy subjects: a combined analysis. Int J Epidemiol. 2012; 41:126–39. [PubMed: 20846947]
40. Smith IM, Mydlarz WK, Mithani SK, Califano JA. DNA global hypomethylation in squamous cell head and neck cancer associated with smoking, alcohol consumption and stage. Int J Cancer. 2007; 121:1724–8. [PubMed: 17582607]
41. Hillemacher T, Frieling H, Moskau S, Muschler MA, Semmler A, Kornhuber J, et al. Global DNA methylation is influenced by smoking behaviour. Eur Neuropsychopharmacol. 2008; 18:295–8. [PubMed: 18242065]
42. Figueiredo JC, Grau MV, Wallace K, Levine AJ, Shen L, Hamdan R, et al. Global DNA hypomethylation (LINE-1) in the normal colon and lifestyle characteristics and dietary and genetic factors. Cancer Epidemiol Biomarkers Prev. 2009; 18:1041–9. [PubMed: 19336559]
43. Zhang FF, Cardarelli R, Carroll J, Fulda KG, Kaur M, Gonzalez K, et al. Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. Epigenetics. 2011; 6:623–9. [PubMed: 21739720]
44. DeMarini DM. Genotoxicity of tobacco smoke and tobacco smoke condensate: a review. Mutat Res. 2004; 567:447–74. [PubMed: 15572290]
45. Wu HC, Delgado-Cruzata L, Flom JD, Kappil M, Ferris JS, Liao Y, et al. Global methylation profiles in DNA from different blood cell types. Epigenetics. 2011; 6:76–85. [PubMed: 20890131]

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TRANSLATIONAL RELEVANCE

Renal cell carcinoma (RCC) is the most lethal genitourinary cancer. Cigarette smoking, hypertension and obesity are the major risk factors for RCC; however, much of the etiology of this disease remains to be elucidated. In this large-scale study, we found that low genomic DNA methylation in peripheral blood was significantly associated with increased risk of RCC. Stratified analyses revealed stronger association in males, younger age at diagnosis, never smokers, late clinical stage, and family history of other cancer indicating combined or interaction effects with traditional risk factors in the etiology of RCC. The results of this study add to the growing body of evidence that hypomethylation of epigenome may be associated with increased cancer risk. Further studies in prospective cohorts are warranted to confirm these results and to apply the findings to clinical assessment of RCC risk.

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Table 1

Characteristics of renal cell carcinoma and control subjects

Variable	Cases n (%)	Controls n (%)	*P* value
Age, mean (SD)	59.29(10.42)	59.39(10.30)	
Pack-years, mean (SD)	24.69(22.01)	25.46(29.52)	0.68
BMI, mean (SD)	29.61(6.13)	28.91(6.56)	0.04
Gender			
Male	596(67.04)	596(67.04)	
Female	293(32.96)	293(32.96)	
Smoking status			
Never	428(50.18)	463(52.20)	
Former	318(37.28)	307(34.61)	
Current	107(12.54)	117(13.19)	0.51
Ethnicity			
Caucasian	754(84.81)	754(84.81)	
Hispanic	77(8.66)	77(8.66)	
African-American	49(5.51)	49(5.51)	
Other	9(1.01)	9(1.01)	
Hypertension			
Yes	490(61.48)	323(41.79)	
No	307(38.52)	450(58.21)	5.81E-15
BMI			
<25	145(22.00)	192(26.34)	
25~30	248(37.63)	298(40.88)	
≥30	266(40.36)	239(32.78)	0.01
BMI at age 20			
<25	427(65.19)	579(79.53)	
25~30	187(28.55)	123(16.90)	
≥30	41(6.26)	26(3.57)	1.70E-08
BMI at age 40			
<25	191(29.43)	351(49.16)	
25~30	282(43.45)	252(35.29)	
≥30	176(27.12)	111(15.55)	6.67E-14
Physical activity			
Low	336(54.19)	205(28.71)	
Medium	185(29.84)	305(42.72)	
Intensive	99(15.97)	204(28.57)	1.48E-20
Family History			
No Cancer	82(10.29)	119(15.41)	
Kidney Cancer	81(10.16)	31(4.02)	
Other Cancer	634(79.55)	622(80.57)	5.42E-07
5-mC% methylation level	Median range)	Median (range)	

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Variable	Cases n (%)	Controls n (%)	*P* value
	3.64(0.28–20.77)	3.97(0.56–21.57)	6.35E-04
Clinical Stage			
I	423(47.58)		
II	49(5.51)		
III	237(26.65)		
IV	165(18.56)		
Histological cell type			
Clear Cell	650(73.11)		
Others	194(21.82)		

BMI, body mass index; 5-mC%, 5-methylcytosine percentage

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Table 2

Predictors of global DNA methylation by univariate general linear model

	Among Controls				Among all participants (cases and controls)			
Variable	**N**	**Mean(SE)**	**β**	***P* value**	**N**	**Mean(SE)**	**β**	***P* value**
Total	889	4.69(0.10)			1778	4.45(0.06)		
Age at diagnosis								
≤60	482	4.81(0.14)			951	4.45(0.09)		
> 60	407	4.54(0.13)	−0.27	0.15	827	4.44(0.09)	−0.01	0.95
Pack-years								
>0 & <30	249	4.89(0.19)			495	4.65(0.13)		
≥30	126	4.42(0.22)	−0.48	0.12	264	4.38(0.17)	−0.27	0.21
Gender								
Male	596	4.64(0.11)			1192	4.41(0.08)		
Female	293	4.80(0.18)	0.16	0.44	586	4.52(0.12)	0.11	0.44
Smoking status								
Never	463	4.80(0.14)			891	4.48(0.09)		
Former	307	4.46(0.15)			625	4.41(0.11)		
Current	117	4.88(0.27)	−0.06	0.64	224	4.64(0.19)	0.04	0.65
Ethnicity								
Caucasian	754	4.68(0.10)			1508	4.47(0.07)		
Hispanic	77	5.29(0.34)			154	4.78(0.24)		
African-American	49	4.22(0.42)			98	3.81(0.24)		
Other	9	2.98(0.65)	−0.15	0.36	18	3.53(0.48)	−0.20	0.07
Hypertension								
Yes	323	4.79(0.16)			813	4.45(0.09)		
No	450	4.95(0.14)	0.16	0.45	757	4.67(0.10)	0.22	0.12
BMI								
<25	192	5.08(0.24)			337	4.75(0.17)		
25~30	298	4.79(0.16)			546	4.56(0.11)		
≥30	239	4.72(0.18)	−0.17	0.23	505	4.46(0.12)	−0.14	0.15
BMI at age 20								

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	Among Controls				Among all participants (cases and controls)			
Variable	**N**	**Mean(SE)**	**β**	***P* value**	**N**	**Mean(SE)**	**β**	***P* value**
<25	579	4.87(0.12)			1006	4.63(0.09)		
25~30	123	4.75(0.26)			310	4.38(0.16)		
≥30	26	4.60(0.53)	−0.13	0.55	67	4.62(0.30)	−0.12	0.36
BMI at age 40								
<25	351	4.94(0.17)			542	4.67(0.13)		
25~30	252	4.90(0.17)			534	4.65(0.12)		
≥30	111	4.30(0.26)	−0.26	0.08	**287**	**4.19(0.15)**	**−0.21**	**0.03**
Physical activity								
Low	205	4.71(0.21)			541	4.45(0.12)		
Medium	305	4.72(0.16)			490	4.50(0.12)		
Intensive	204	5.00(0.20)	0.14	0.31	**303**	**4.89(0.16)**	**0.20**	**0.03**
Family History								
No Cancer	119	5.02(0.30)			201	4.79(0.22)		
Kidney Cancer	31	4.20(0.52)			112	4.31(0.25)		
Other Cancer	622	4.89(0.12)	−0.02	0.87	1256	4.54(0.08)	−0.08	0.40
Clinical Stage								
I					423	4.19(0.12)		
II					49	3.78(0.32)		
III					237	4.01(0.17)		
IV					165	4.74(0.21)	0.10	0.15
Histological cell type								
Clear Cell					650	4.30(0.10)		
Others					194	4.08(0.17)	−0.22	0.30

BMI, body mass index; 5-mC%, 5-methylcytosine percentage

Results in bold were significant predictors of global methylation by univariate general linear model

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Table 3

Association between global DNA methylation levels classified by the tertiles of the distribution of controls and renal cell carcinoma risk

5-mC%	Cases n (%)	Controls n (%)	OR[a]	95% CI	*P* value	OR[b]	95% CI	*P* value
T3	247(27.78)	296(33.30)	1(Ref)			1(Ref)		
T2	286(32.17)	294(33.07)	1.18	(0.92–1.50)	0.19	1.30	(0.98–1.72)	0.07
T1	356(40.04)	299(33.63)	1.46	(1.15–1.84)	1.64E-03	1.40	(1.06–1.84)	0.02
p for trend					1.49E-03			0.02

5-mC%, 5-methylcytosine percentage

[a]Conditional logistic regression model adjusting for gender, age and ethnicity

[b]Conditional logistic regression model adjusting for gender, age, ethnicity, smoking status, BMI, hypertension, physical activity and family cancer history

T3, third tertile of methylation (≥5.25); T2, second tertile of methylation (3.1–5.24); T1, first tertile of methylation (≤3.1)

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Table 4

Risk of RCC estimated by global DNA methylation stratified by selected host characteristics

Group	5-mC%	Cases n (%)	Controls n (%)	OR[a]	95% CI	*P* value	OR[b]	95% CI	*P* value
Age at diagnosis									
≤60									
	T3	126(26.87)	164(34.02)	1(Ref)			1(Ref)		
	T2	142(30.28)	159(32.99)	1.16	(0.83–1.60)	0.38	1.13	(0.79–1.62)	0.50
	T1	201(42.86)	159(32.99)	1.63	(1.19–2.24)	2.17E-03	1.47	(1.04–2.08)	0.03
	p for trend					1.81E-03			0.03
>60									
	T3	121(28.81)	132(32.43)	1(Ref)			1(Ref)		
	T2	144(34.29)	135(33.17)	1.16	(0.83–1.64)	0.38	1.09	(0.75–1.59)	0.65
	T1	155(36.90)	140(34.40)	1.22	(0.87–1.70)	0.26	1.22	(0.84–1.78)	0.30
	p for trend					0.27			0.30
	p for interaction								
Gender									
Male									
	T3	164(27.52)	197(33.05)	1(Ref)			1(Ref)		
	T2	184(30.87)	210(35.23)	1.06	(0.79–1.42)	0.70	1.17	(0.83–1.65)	0.38
	T1	248(41.61)	189(31.71)	1.59	(1.19–2.12)	1.74E-03	1.61	(1.14–2.28)	0.01
	p for trend					1.10E-03			0.01
Female									
	T3	83(28.33)	99(33.79)	1(Ref)			1(Ref)		
	T2	102(34.81)	84(28.67)	1.53	(0.99–2.36)	0.06	1.68	(0.98–2.87)	0.06
	T1	108(36.86)	110(37.54)	1.20	(0.80–1.80)	0.38	1.02	(0.62–1.70)	0.93
	p for trend					0.42			0.99
	p for interaction								
Smoking Status									
Never									
	T3	120(28.04)	151(32.61)	1(Ref)			1(Ref)		
	T2	138(32.24)	166(35.85)	1.03	(0.74–1.44)	0.85	1.07	(0.75–1.54)	0.70

Group	5-mC%	Cases n (%)	Controls n (%)	OR[a]	95% CI	P value	OR[b]	95% CI	P value
	T1	170(39.72)	146(31.53)	1.47	(1.06–2.04)	0.02	1.55	(1.08–2.23)	0.02
	p for trend					0.02			0.02
Former									
	T3	91(28.62)	99(32.25)	1(Ref)			1(Ref)		
	T2	102(32.08)	95(30.94)	1.15	(0.77–1.73)	0.49	1.04	(0.67–1.62)	0.86
	T1	125(39.31)	113(36.81)	1.18	(0.80–1.74)	0.40	1.11	(0.73–1.70)	0.63
	p for trend					0.41			0.63
Current									
	T3	32(29.91)	45(38.46)	1(Ref)			1(Ref)		
	T2	35(32.71)	33(28.21)	1.48	(0.75–2.92)	0.26	1.37	(0.63–2.94)	0.43
	T1	40(37.38)	39(33.33)	1.43	(0.75–2.73)	0.28	1.15	(0.54–2.43)	0.72
	p for trend					0.30			0.75
	p for interaction								
Family History									
No cancer									
	T3	25(30.49)	50(42.02)	1(Ref)			1(Ref)		
	T2	23(28.05)	28(23.53)	1.58	(0.75–3.32)	0.23	1.53	(0.68–3.44)	0.30
	T1	34(41.46)	41(34.45)	1.56	(0.79–3.06)	0.20	1.24	(0.58–2.62)	0.58
	p for trend					0.20			0.57
Kidney Cancer									
	T3	27(33.33)	8(25.81)	1(Ref)			1(Ref)		
	T2	21(25.93)	10(32.26)	0.91	(0.29–2.85)	0.88	0.62	(0.14–2.69)	0.52
	T1	33(40.74)	13(41.94)	0.94	(0.32–2.76)	0.91	0.89	(0.21–3.77)	0.87
	p for trend					0.92			0.93
Other Cancer									
	T3	174(27.44)	221(35.53)	1(Ref)			1(Ref)		
	T2	215(33.91)	217(34.89)	1.26	(0.95–1.65)	0.10	1.25	(0.93–1.68)	0.15
	T1	245(38.64)	184(29.58)	1.69	(1.28–2.24)	1.89E-04	1.64	(1.21–2.20)	1.25E-03
	p for trend					1.83E-04			1.22E-03
	p for interaction								
Clinical Stage									

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Group	5-mC%	Cases n (%)	Controls n (%)	OR[a]	95% CI	*P* value	OR[b]	95% CI	*P* value
Early Stage (I/II)									
	T3	64(28.83)	296(33.30)	1(Ref)			1(Ref)		
	T2	62(27.93)	294(33.07)	1.02	(0.69–1.50)	0.92	1.00	(0.66–1.49)	0.98
	T1	96(43.24)	299(33.63)	1.51	(1.06–2.16)	0.02	1.46	(1.00–2.14)	0.05
	p for trend					0.02			0.05
Late Stage (III/IV)									
	T3	53(27.89)	296(33.30)	1(Ref)			1(Ref)		
	T2	49(25.79)	294(33.07)	0.94	(0.62–1.43)	0.77	1.07	(0.68–1.68)	0.77
	T1	88(46.32)	299(33.63)	1.69	(1.16–2.48)	0.01	2.06	(1.36–3.12)	7.04E-04
	p for trend					4.17E-03			4.98E-04
Histological cell type									
Clear cell type									
	T3	189(29.17)	296(33.30)	1(Ref)			1(Ref)		
	T2	198(30.56)	294(33.07)	1.08	(0.83–1.39)	0.58	1.06	(0.80–1.41)	0.69
	T1	261(40.28)	299(33.63)	1.38	(1.08–1.77)	0.01	1.38	(1.05–1.82)	0.02
	p for trend					0.01			0.02
Other cell type									
	T3	48(24.74)	296(33.30)	1(Ref)			1(Ref)		
	T2	75(38.66)	294(33.07)	1.47	(0.99–2.20)	0.06	1.54	(1.01–2.35)	0.05
	T1	71(36.60)	299(33.63)	1.41	(0.94–2.10)	0.10	1.47	(0.95–2.27)	0.08
	p for trend					0.11			0.09

5-mC%, 5-methylcytosine percentage

[a]Conditional logistic regression model adjusting for gender, age and ethnicity

[b]Conditional logistic regression model adjusting for gender, age, ethnicity, smoking status, BMI, hypertension, physical activity and family cancer history

T3, third tertile of methylation (≥5.25); T2, second tertile of methylation (3.1–5.24); T1, first tertile of methylation (≤3.1)