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BPDE-Induced Lymphocytic Chromosome 3p Deletions May Predict Renal Cell Carcinoma Risk

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Abstract

Purpose—Cigarette smoking is a risk factor for renal cell carcinoma (RCC). Benzo[*a*]pyrene diol epoxide (BPDE), a major constituent of cigarette smoke, induces 3p aberrations that are associated with susceptibility to other smoking-associated cancers. Because chromosome 3p deletions are known to be the most frequent genetic alterations in RCC, we tested whether 3p sensitivity to BPDE predicted susceptibility to RCC.

Material and Methods—Cultured peripheral blood lymphocytic cells from 170 cases and 135 control subjects were treated with 2 μ M BPDE for 24 hours and assessed for 3p deletions by fluorescence *in situ* hybridization using probes directed to 3p25.2, 3p21.3, 3p14.2, and 3p12.2; a probe for 3q13 was used as a control. One thousand lymphocyte interphases were scored for each sample.

Results—At each locus, BPDE-induced 3p deletions were significantly more frequent in cases than in controls. No significant differences between cases and controls were observed for deletions in 3q13. Using the median value in the controls as the cutoff point for BPDE sensitivity, we found the odds ratios for subjects with high BPDE sensitivity at 3p25.2, 3p21.3, 3p14.2, and 3p12.2 were 2.02 (95% confidence interval [CI], 1.15–3.37), 2.28 (95% CI, 1.07–3.16), 1.84 (95% CI, 1.33–3.92), and 1.97 (95% CI, 1.18–3.46), respectively. There were dose-dependent relationships between the number of deletions at each locus and risk for RCC.

Conclusions—This study demonstrated that chromosome 3p may be a specific molecular target of cigarette carcinogens and that BPDE sensitivity in chromosome 3p may reflect an individual's genetic susceptibility to RCC.

Keywords

Renal cell carcinoma; BPDE; chromosome 3p; smoking; lymphocyte

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Introduction

Renal cell carcinoma (RCC) accounts for around 3% of human malignancies and is the 10th leading cause of male cancer death in the United States.¹ Most RCCs detected as an incidental mass can be treated surgically but it is difficult to attain complete cure in the case of advanced RCCs even when using the latest multimodal therapy. Therefore, early detection and prevention of this cancer are of paramount importance. The association between cigarette smoking and RCC risk was established by an International Agency for Research on Cancer (IARC) Working Group in 2002.^{2,3} Furthermore, the recent meta-analysis of 24 previous studies revealed the significant association between cigarette smoking and increased risk of RCC.⁴ While the association between smoking and RCC is established, the genetic mechanisms through which tobacco smoke affects RCC development remain unknown. Identifying specific molecular targets affected by carcinogens in tobacco might provide insight into RCC carcinogenesis as well as potential inherited susceptibility.

Previous studies have shown that smoking-associated cancers, such as lung cancer and squamous cell carcinoma of the head and neck (HNSCC), are frequently associated with chromosome 3p aberrations,^{5–8} suggesting that chromosome 3p is an important target region for tobacco carcinogens. Chromosome 3p aberrations are also the most frequently identified genetic alterations in RCC.^{9–12} Recent cytogenetic studies have shown that, besides a 3p25 region, which contains the *VHL* tumor suppressor gene (TSG), the 3p21.3 and 3p14.2 regions were frequently deleted in RCC, suggesting that they may contain TSGs, too.^{10–14} Taken together, these evidences suggest that chromosome 3p is the most important genetic region in RCC carcinogenesis.

BPDE is the metabolic product of benzo[*a*]pyrene, a major constituent of tobacco smoke, and has been tested in numerous epidemiological studies examining mutagen sensitivity.¹³ Our previous studies of lung cancer⁵ and HNSCC⁷ revealed BPDE-induced 3p21.3 aberrations more frequently in PBLs from patients than in those from healthy controls; we also showed that measuring these aberrations had the potential to predict an individual's genetic susceptibility to smoking-associated cancers. However, the predictive utility of such testing has not yet been reported in RCC. In this study, we hypothesized that quantification of BPDE-induced chromosome 3p aberrations may be useful in predicting a person's risk of RCC. To test this hypothesis, we performed assays for BPDE-induced sensitivity using locus-specific fluorescent in situ hybridization (FISH) probes for 3p25.2, 3p21.3, 3p14.2, and 3p12.2 in PBLs from patients with RCC and healthy controls.

Materials and methods

Study subjects

We identified 170 newly diagnosed, histologically confirmed RCC from The University of Texas M. D. Anderson Cancer Center. No restrictions were placed on patient age, gender, ethnicity, or on tumor stage. A detailed description of control subject recruitment was detailed elsewhere.¹⁴ Briefly, the 135 control subjects without a history of cancer were enrolled from Kelsey-Seybold Clinic, the largest multispecialty health care organization in Houston area, during the same time period as patients. Most controls visited these clinics for their checkups, not treatment of chronic illnesses. The control subjects were frequency matched to the patients by age (± 5 years), gender, and ethnicity. Each participant was asked to sign an informed consent form for the blood drawing and for completion of a personal interview. The study was approved by the Institutional Review Boards of M.D. Anderson Cancer Center and Kelsey-Seybold Clinic.

Epidemiologic data collection

The personal interview was conducted using a questionnaire to collect comprehensive epidemiologic data regarding demographic data, smoking status and family history of cancer. Blood was drawn into sodium heparinized tubes for cytogenetic and molecular genetic analyses following the interview. The personnel who performed the laboratory analyses were blinded with regard to case-control status.

BPDE sensitivity assay

BPDE-induced lymphocytic chromosome aberrations were measured as previously described.⁷ Briefly, lymphocyte cultures were set up following the routine protocol of adding 1 ml of whole blood to 9 mL RPMI 1640 tissue culture medium (JRM Biosciences, Lenexa, KS) with 10% fetal calf serum and 1% phytohemagglutinin (Wellcome Research Laboratories, Research Triangle Park, NC) at 37 °C for 48 hours. Each culture was treated with BPDE (Midwest Research Institute, Kansas City, MO) to a final concentration of 2 µM for 24 hours. After routine blocking with colcemid, hypotonic treatment, and fixation, cell suspensions were stored at -20 °C until they were processed for chromosome detection by FISH. DNA probes for FISH at 3p25.2, 3p21.3, 3p14.2 and 3p12.2, and 3q13 (a control probe) were obtained from Oncor (Gaithersburg, MD). 3q13 was used as an internal control because 3q13 has not been reported to be deleted in RCC, furthermore, the 3q13 probe targets a locus located in a Giemsa-light region, similar to 3p21.3. FISH with each probe was performed according to the supplier's instructions. BPDE-treated and untreated cells were taken from the cell suspensions and then 10µl of probe was added to each interphase smear slide. The slides were pretreated with 2× SSC, dehydrated, and denatured. Hybridization was performed at 37°C for at least 16 hours and fluorescent signals were detected by incubating the slides in fluorescein isothiocyanate (FITC)-conjugated avidin (Oncor). The slides were then incubated with FITC-anti-avidin antibody. Finally, the slides were counterstained with 4'-diamino-2-phenylindole (DAPI) and the cells were viewed through a fluorescence microscope with individual FITC filters. We scored 1,000 interphase cells for each sample to detect and enumerate chromosomal aberrations. The criteria for scoring FISH signals were the same as previously described.^{5, 7} Only non-overlapped nuclei with clearly separated fluorescent signals were scored. Minor hybridization spots, which are smaller and less intense than real signals, were excluded. Paired or close signals were counted as one signal. The laboratory personnel who did the scoring were blinded to the sample's case-control status.

Statistical analysis

All statistical analyses were performed using Stata statistical software (Stata Corp., College Station, TX). Pearson's chi-square was used to test for significance of differences in the distribution between cases and controls. We examined the association between 3p BPDE sensitivity, as indicated by the number of 3p25.2, 3p21.3, 3p14.2, and 3p12.2 aberrations per 1000 interphases, and the risk of RCC. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated as estimates of relative risk. ORs were calculated by logistic regression adjusted for multiple covariates.

Results

The characteristics of the study subjects are summarized in table 1. This case-control study comprised 170 cases and 135 controls. There were no significant differences between the cases and controls in terms of age, gender, ethnicity, and smoking status. In terms of tumor histology, 134 patients (78.8%) had conventional (clear cell) RCC, 18 (10.6%) had papillary type, 6 (3.5%) had chromophobe type, 3 (1.8%) had sarcomatoid type, and 9 (5.2%) had unclassified type.

Two FISH signals represented two copies of the chromosome region in a normal cell. A deletion was defined as one signal in an interphase cell.^{5,7} Cases exhibited significantly more BPDE-induced deletions (per 1000 interphase cells) than controls at the 3p25.2 (29.1 ± 8.79 vs. 25.4 ± 7.89 , $p < 0.001$), 3p21.3 (35.9 ± 8.78 vs. 31.8 ± 7.14 , $p < 0.001$), 3p14.2 (23.6 ± 8.49 vs. 19.8 ± 6.95 , $p < 0.001$), and 3p12.2 (21.4 ± 8.00 vs. 18.6 ± 6.37 , $p = 0.003$) loci (Fig. 1). At the 3q13 locus, there were no statistically significant differences between case and control subjects (6.59 ± 3.21 vs. 6.51 ± 2.72 , $p = 0.84$). To assess RCC risk associated with BPDE sensitivity, we dichotomized subjects into low and high BPDE sensitivity based on the median number of deletions at each locus in controls. After adjusting for age, gender, ethnicity, and smoking status, we found the ORs for subjects with high BPDE sensitivity were 2.02 (95% CI, 1.15–3.37) at 3p25.2, 2.28 (95% CI, 1.07–3.16) at 3p21.3, 1.84 (95% CI, 1.33–3.92) at 3p14.2 and 1.97 (95% CI, 1.18–3.46) at 3p12.2 (table 2). The OR for 3q13 was 0.92 (95% CI, 0.51–1.67). In quartile analysis, using the lowest quartile (that is, subjects showing the least sensitivity to BPDE) as the reference group, the ORs associated with deletions at 3p25.2 for subjects in the 2nd, 3rd, and 4th quartiles were 1.15 (95% CI, 0.47–2.78), 1.63 (95% CI, 0.68–3.90) and 2.80 (95% CI, 1.20–6.57), respectively (p for trend = 0.004; table 3). Similar trends were observed for ORs associated with deletions at 3p21.3 (p for trend = 0.002), 3p14.2 (p for trend = 0.003), and 3p12.2 (p for trend = 0.003) (table 3).

When subjects were stratified by smoking status (never or ever smoker), never smokers in the high-BPDE-sensitivity group exhibited significantly higher risks than those in the low-sensitivity group, with ORs of 4.12 (95% CI, 1.80–9.44) at 3p25.2, 3.16 (95% CI, 1.40–7.15) at 3p21.3 and 2.94 (95% CI, 1.27–6.81) at 3p14.2 (table 4). However, in ever smokers, no significant increase in risk was associated with an increase in deletions at any locus (table 4). We also analyzed the interactions of BPDE sensitivity at each locus and smoking status (never or ever smoker) (table 5). There was a significant interaction between deletions at 3p25.2 locus and smoking status (p for interaction = 0.012).

Discussion

Results from our analysis of BPDE sensitivity in the PBLs of patients with RCC and matched controls support our hypothesis that BPDE-induced deletions of various 3p loci may predict susceptibility to RCC. This result is consistent with those from our previous studies showing that BPDE induced 3p aberrations in smoking-associated cancer.^{5,7} This result also provides a biological explanation for smoking as a risk factor for RCC.

In our study the greatest increase in risk of RCC occurred in patients with high BPDE sensitivity at 3p21.3 (OR = 2.28; 95% CI, 1.33–3.92). This risk was somewhat lower than those in our previous studies showing 3p sensitivity to BPDE in other smoking-associated cancers. Using the same experimental procedure, we found that a lung cancer exhibited a 14.1-fold (95% CI, 3.5–56.2) increased risk⁵ and HNSCC a 4.8-fold (95% CI, 1.87–12.28) increased risk,⁷ although these two studies evaluated only a small number of subjects. Although RCC is associated with smoking, smoking's effect on RCC tumorigenesis is not as strong as it is in the major smoking-associated cancers. According to the 2002 report of the IARC working group based on 21 epidemiology studies, heavy smokers (those smoking more than 20 cigarettes/day) had mean increase in RCC risk of 1.5 to 2.0 times on average that of never smokers.^{2,3} In contrast, increased risks in the major smoking-associated cancers, such as those of the lung and urinary tract, have been shown to be more than two times higher than those in RCC.^{2,3} Thus, the results from this study and earlier studies^{5,7} are consistent with the epidemiological evidence that cigarette smoking is a modest risk factor of RCC.

Our previous studies showed that the 3p21.3 locus was specifically prone to BPDE-induced aberrations.^{5,7} In the present study, we examined BPDE-induced deletions at four loci

spanning chromosome 3p, and we found that all four loci were more sensitive to BPDE in patients than in controls. The number of 3p21.3 deletions was somewhat higher than those of other loci (Fig. 1). This result appears to be similar to previous findings that 3p21.3 showed the highest frequency of loss of heterozygosity (LOH) in RCC tissues. These results also imply the existence of several TSGs near this locus.^{9, 12} One study examining 3p allelic status with microsatellite markers showed that most conventional RCCs had a terminal LOH at 3p14.2-25, and RCC of other histologic types, such as papillary and chromophobe RCCs, also had LOH on 3p in the same pattern but at a somewhat lower frequency.¹¹ Another study using comparative genetic hybridization has suggested that these chromosome 3p losses occur in several specific regions: 3p25-26, 3p21.3-22, 3p21.2 and 3p13-14.¹² Our results support these cytogenetic findings of the extensive deletions on chromosome 3p in RCC. Previous studies of lung cancer have implicated that 3p21 and 3p14.2 were putative targets of tobacco carcinogen. Indeed, 3p14.2 include the most common fragile site FRA3B, and the frequency of LOH at this locus was shown to be markedly higher in smokers than in non-smokers.⁶ Although 3p LOH occurred in most conventional RCCs, the effect of smoking on RCC incidence is not so strong compared with lung cancer.^{3, 4} We speculate that in RCC, there may be alternative major mechanisms to cause 3p alteration in addition to tobacco carcinogens. Fragile site at 3p14.2 may be one of the alternative mechanisms.

Because it is difficult to obtain large numbers of cultured tissue cells, we used PBLs as surrogate tissues. Previous studies have shown concordant aberrations of specific chromosome regions in lymphocytes and tumor tissues in other cancers.^{15–17} Chromosome 3p deletions occur in most conventional RCCs. We also have used high-density SNP array CGH to profile chromosome aberrations in tumors from a subset of these patients and found that a large fragment or whole 3p deletion occurred in roughly 85% of conventional RCCs (data now shown). It appears that 3p is a potential molecular target of tobacco carcinogens. Nevertheless, further studies are warranted to determine that BPDE directly induce 3p deletions in RCC tissues.

It is interesting that our stratified analyses by smoking status showed that only never smokers with high sensitivity to BPDE at 3p14.2, 3p21.3, and 3p25.2 loci exhibited an increased risk of RCC, whereas no significant increase in risk was found in ever smokers for deletions at any locus. The finding that never smokers had higher sensitivity to BPDE suggest that ever smokers have more proficient DNA repair capacity, stimulated by chronic DNA damage due to tobacco carcinogens. This observation is consistent to a recent study examining the association between mutagen sensitivity and lung cancer risk. In that study, current smokers exhibited the lowest number of chromosome breaks induced by bleomycin or BPDE as compared with never or former smokers.¹⁸

Losses of chromosome 3p—especially at 3p25 locus, which harbors the *VHL* gene—have been reported to be early genetic alterations in conventional (clear cell) RCC. While 3p losses reportedly occur in 80% to 98% of sporadic clear cell RCC cases and are evident at all disease stages,^{9–12} the *VHL* gene mutation and promoter hypermethylation have been reported in 52% to 70%, and 5% to 20% of cases, respectively.^{19, 20} Results from a recent prospective cohort study indicated that smoking was associated with an increased risk of clear cell RCC in men (RR = 2.54, 95% CI, 1.05–6.17), but there was no significant association between *VHL* mutations and smoking status, suggesting that smoking may cause clear cell RCC independent of *VHL* gene mutations.²⁰ Taken together, these previous findings and our current results suggest that chromosome 3p deletions induced by tobacco carcinogens might be an initial step in the carcinogenesis of clear cell RCC.

Conclusions

Chromosome 3p may be a molecular target for BPDE damage in RCC, as is in other smoking-associated cancers. The degree of BPDE sensitivity at chromosome 3p may be a suitable marker for an individual's predisposition to RCC. To the best of our knowledge, this study is the first epidemiologic study to explore the association between smoking and RCC risk cytogenetically.

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Abbreviations

RCC	renal cell carcinoma
BPDE	benzo[α]pyrene diol epoxide
PBL	peripheral blood lymphocyte
FISH	fluorescent in situ hybridization
TSG	Tumor suppressor gene

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Fig. 1.
Frequencies of chromosome 3p deletions at each locus examined in the BPDE-sensitivity assay in patients with RCC and matched control subjects.

Table 1

Distribution of host characteristics in patients and control subject

Variable	Cases (<i>n</i> = 170)	Controls (<i>n</i> = 135)	p value
Age, Mean ($\pm$ SD)	59.0 (10.4)	59.1 (10.4)	0.90
Gender, <i>n</i> (%)			
Male	109 (64.1)	86 (63.7)	0.94
Female	61 (35.9)	49 (36.3)	
Ethnicity, <i>n</i> (%)			
Caucasian	121 (71.2)	105 (77.8)	0.15
Mexican American	33 (19.4)	15 (11.1)	
African American	11 (6.5)	13 (9.6)	
Others	5 (2.9)	2 (1.5)	
Smoking Status, <i>n</i> (%)			
Never	78 (47.3)	61 (45.2)	0.69
Former	61 (37.0)	56 (41.5)	
Current	26 (15.8)	18 (13.3)	

Table 2

Risk estimates for BPDE-induced 3p deletions in patients with RCC and healthy controls

	No. (%)		Adjusted OR (95% CI) [*]
	Cases	Controls	
3p25.2			
Low sensitivity	44 (34.1)	49 (47.6)	1
High sensitivity [†]	85 (65.9)	54 (52.4)	2.02 (1.18–3.46)
3p21.3			
Low sensitivity	44 (29.7)	50 (46.3)	1
High sensitivity [†]	104 (70.3)	58 (53.7)	2.28 (1.33–3.92)
3p14.2			
Low sensitivity	43 (31.9)	48 (45.3)	1
High sensitivity [†]	92 (68.1)	58 (54.7)	1.84 (1.07–3.16)
3p12.2			
Low sensitivity	41 (29.9)	50 (46.7)	1
High sensitivity [†]	96 (70.1)	57 (53.3)	1.97 (1.15–3.37)
3q13 (control)			
Low sensitivity	40 (41.7)	43 (40.5)	1
High sensitivity [†]	56 (58.3)	63 (59.5)	0.92 (0.51–1.67)

^{*} Odds ratio (OR) adjusted for age (as a continuous variable), gender, ethnicity, and smoking status; data are means and 95% confidence intervals (CI)

[†] Categorized at the median value of deletions in the control group

Not all patients and controls had completed analysis for deletions at each locus

Table 3

Risk estimate for BPDE-induced 3p deletions with subjects grouped according to the quartile distribution in the controls

	No. (%)		Adjusted OR (95% CI)*	p for trend
	Cases	Controls		
3p25.2 deletion				
1st quartile	16 (11.5)	18 (17.5)	Reference	0.004
2nd quartile	28 (20.1)	31 (30.1)	1.15 (0.47–2.78)	
3rd quartile	25 (25.2)	27 (26.2)	1.63 (0.68–3.90)	
4th quartile	60 (43.2)	27 (26.2)	2.80 (1.20–6.57)	
3p21.3 deletion				
1st quartile	18 (12.2)	24 (22.2)	Reference	0.002
2nd quartile	26 (17.6)	26 (24.1)	1.42 (0.61–3.30)	
3rd quartile	43 (29.0)	27 (25.0)	2.48 (1.11–5.57)	
4th quartile	61 (41.2)	31 (28.7)	3.04 (1.39–6.64)	
3p14.2 deletion				
1st quartile	7 (5.2)	24 (22.6)	Reference	0.003
2nd quartile	36 (26.7)	24 (22.6)	5.04 (1.82–13.9)	
3rd quartile	40 (29.6)	28 (26.4)	5.09 (1.91–13.6)	
4th quartile	52 (38.5)	30 (28.4)	5.95 (2.21–16.0)	
3p12.2 deletion				
1st quartile	21 (15.3)	20 (18.6)	Reference	0.028
2nd quartile	20 (14.6)	30 (28.0)	0.64 (0.27–1.49)	
3rd quartile	44 (32.1)	30 (28.0)	1.31 (0.59–2.90)	
4th quartile	52 (38.0)	27 (25.2)	1.78 (0.81–3.89)	

* Odds ratio (OR) adjusted for age (as a continuous variable), gender, ethnicity, and smoking status; data are means and 95% confidence intervals (CI)

Not all patients and controls had completed analysis for deletions at each locus

Table 4

Risk estimates for BPDE-induced 3p deletion stratified by smoking status

	Adjusted OR (95% CI) [*]			
	3p25.2	3p21.3	3p14.2	3p12.2
<i>Smoking status</i>				
Never smoker				
Low sensitivity	Reference	Reference	Reference	Reference
High sensitivity	4.12 (1.80 – 9.44)	3.16 (1.40 – 7.15)	2.94 (1.27 – 6.81)	2.17 (0.94 – 5.05)
Ever smoker				
Low sensitivity	Reference	Reference	Reference	Reference
High sensitivity	1.00 (0.48 – 2.10)	1.80 (0.87 – 3.71)	1.26 (0.60 – 2.62)	1.83 (0.91 – 3.71)

* Odds ratio (OR) adjusted for age (as a continuous variable), gender, and ethnicity; data are means and 95% confidence intervals (CI)

Table 5
Joint analysis of BPDE sensitivity and smoking status according to deletions at each 3p locus

BPDE sensitivity	Smoking status	No. (%)		Adjusted OR (95% CI)*	p for interaction
		Cases	Controls		
3p25.2					
Low	Never	15 (11.3)	26 (25.2)	Reference	
High	Never	50 (37.6)	22 (21.4)	4.30 (1.88 – 9.83)	
Low	Ever	27 (20.3)	23 (22.3)	2.38 (0.98 – 5.79)	
High	Ever	41 (30.8)	32 (31.1)	2.46 (1.09 – 5.56)	0.012
3p21.3					
Low	Never	17 (12.0)	25 (23.1)	Reference	
High	Never	47 (33.1)	23 (21.3)	3.24 (1.45 – 7.27)	
Low	Ever	24 (16.9)	25 (23.2)	1.54 (0.66 – 3.59)	
High	Ever	54 (38.0)	35 (32.4)	2.65 (1.21 – 5.78)	0.247
3p14.2					
Low	Never	18 (13.7)	26 (24.5)	Reference	
High	Never	42 (32.1)	22 (20.8)	2.94 (1.29 – 6.67)	
Low	Ever	23 (17.6)	22 (20.8)	1.58 (0.68 – 3.70)	
High	Ever	48 (36.6)	36 (33.9)	2.00 (0.95 – 4.23)	0.133
3p12.2					
Low	Never	15 (11.2)	18 (16.8)	Reference	
High	Never	49 (36.6)	28 (26.2)	2.08 (0.90 – 4.76)	
Low	Ever	26 (19.4)	32 (29.9)	0.94 (0.39 – 2.24)	
High	Ever	44 (32.8)	29 (27.1)	1.76 (0.76 – 4.08)	0.851

* Odds ratio (OR) adjusted for age (as a continuous variable), gender, ethnicity, and smoking status; data are means and 95% confidence intervals (CI)

Not all patients and controls had completed analysis for deletions at each locus