Inducible Nitric Oxide Synthase Promoter Polymorphism, Cigarette Smoking, and Urothelial Carcinoma Risk

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OBJECTIVES Bladder carcinoma has a high inducible nitric oxide synthase (iNOS) content, and a highly polymorphic (CCTTT)n repeat in the *iNOS* promoter region has been identified. We explored whether this *iNOS* promoter polymorphism and cigarette smoking are associated with urothelial carcinoma (UC) risk.

METHODS A total of 250 patients with pathologically confirmed UC and 250 unrelated noncancer controls were serially recruited at the Chia Yi Christian Hospital from August 2002 to May 2005. Multivariate logistic regression analysis was used to calculate the odds ratio and 95% confidence interval (CI).

RESULTS A significantly increased UC risk was found in those who had smoked more than 30 years (odds ratio 2.4, 95% CI 1.5 to 4.2). The study subjects carrying the 12-repeat allele had a significantly increased UC risk (odds ratio 1.7, 95% CI 1.1 to 2.5). We also found the investigated polymorphism was related to clinical stage ($P = 0.043$). Of those who had ever smoked, those with the short/long (S/L) and long/long (L/L) genotypes (S, 9 to 11 repeats; L, 12 to 18 repeats) and the 12-repeat allele had a significantly increased UC risk of 3.5 (95% CI 1.7 to 7.3) and 4.5 (95% CI 2.2 to 8.9), respectively. Of the study subjects who had smoked longer than 30 years, those with S/L and L/L genotypes and the 12-repeat allele had significantly increased UC risks of 2.4 (95% CI 1.3 to 4.7) and 3.8 (95% CI 1.8 to 8.0), respectively.

CONCLUSIONS These findings suggest that the polymorphic (CCTTT)n repeat in the *iNOS* promoter region might be involved in the development of UC, especially in those who have ever smoked. UROLOGY 69: 1001–1006, 2007. © 2007 Elsevier Inc.

Urothelial carcinoma (UC) is the second most common cancer and second leading cause of death among malignancies of the genitourinary tract system.[1] In general, several types of urothelial carcinoma (bladder, renal pelvis, and ureter) have histologic features similar to that of transitional cell carcinoma and are considered to have an analogous etiology.[2] According to the annual report of the Taiwan Cancer Registry in 2001, the age-standardized incidence per 100,000 person-years of bladder cancer was 10.15 in males and 4.02 in females.[3,4] The etiology of UC is heterogeneous, involving ethnic, environmental, genetic, and dietary factors.[5] Cigarette smoking, occupational exposures, inflammatory reactions to parasites or chronic infections, and exposure to arsenic in the drinking water are known to be risk factors for bladder cancer.[6] In particular, cigarette smoke contains polycyclic aromatic hydrocarbons, which might contribute to the etiology of bladder cancer and result in a two to fourfold risk among those who have ever smoked.[7]

Nitric oxide (NO) is a multifunctional gaseous molecule and a reactive free radical that has been shown to play an important role in several diseases, such as vascular disease, immunologic reactions, and cancer.[8,9] It is mainly generated by a family of nitric oxide synthases (NOSs) and is produced by many cell types.[10] NO is a product of the conversion of L-arginine to L-citrulline by NOS.[11] The three NOS isoforms are neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3).[12] The activity of nNOS and eNOS is dependent on the concentration of cytosolic calcium. However, iNOS is independent of the intracellular calcium levels and produces more NO than nNOS and eNOS.[13] Overexpression of iNOS has been reported in several human cancers, including bladder cancer, and continuous NO production might be

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involved in the inflammatory process associated with many malignancies.[14,15] Some studies have found that iNOS levels are greater in malignant tissue, and the localization of iNOS to the intratumoral macrophages and endothelial cells of the tumor vasculature suggests that the intratumoral microenvironment is conducive to the induction of iNOS.[16,17] Benzo[a]pyrene is a carcinogen found in cigarettes. It results in oxidant stress, which leads to the stimulation of iNOS.[18] The overexpression of iNOS is mainly regulated by several polymorphisms, which have been identified in the *iNOS* promoter. A polymorphic pentanucleotide (CCTTT)n repeat approximately 2.5 kilobases upstream the transcription initiation site has been elucidated to affect iNOS expression.[19] Although the overexpression of NOS occurs in various tumor cell lines and solid tumors, the role of NO in tumor biology is still unclear. In this study, we explored the association among the polymorphic (CCTTT)n repeat in the *iNOS* promoter, cigarette smoking, and UC risk.

MATERIAL AND METHODS

Study Subject Selection

A case-control study of UC (bladder, renal pelvis, and ureter) was conducted at the Chia Yi Christian Hospital. A total of 250 patients with UC (mean age 64.6 ± 12.7 years) were serially recruited from the Department of Urology. All cases of UC had been histologically confirmed from August 2002 to May 2005. Patients with recurrent UC or those who received intravesical bacille Calmette-Guérin (BCG) instillations, radiotherapy, or chemotherapy preoperatively were excluded. All the pathologic types were transitional cell carcinoma. One experienced pathologist reviewed all pathology information. The staging and grading of tumors was done according to the criteria of the TNM staging system and the World Health Organization International Society of Urological Pathology.[20] The control group consisted of 250 unrelated subjects (mean age 62.1 ± 12.4 years), who were patients admitted to the same hospital, with an absence of any cancer history or precancerous lesions. All subjects were interviewed by a trained interviewer using a standard questionnaire. The collected information included: (a) demographic characteristics, (b) dietary habits, (c) a history of cigarette smoking and alcohol drinking, and (d) a history of other diseases. Ten milliliters of venous blood was collected into vials from all subjects. Genomic DNA was extracted from the peripheral blood lymphocytes using proteinase K and phenol chloroform. The institutional review board at the Chia Yi Christian Hospital approved this study, and all study subjects provided informed consent.

Genotyping of *iNOS* Promoter Polymorphism

The genotyping was determined using polymerase chain reaction with the following primers: 5′-ACC CCT GGA AGC CTA CAA CTG CAT-3′ (sense); 5′-GCC ACT GCA CCC TAG CCT GTC TCA-3′ (antisense). The sense primer was labeled with HEX dye. The thermal cycling was performed with an initial step at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 20s, and a final extension at 72°C for 10 min. Genotyping was performed in a mixture of amplified products using an internal size standard by ABI PRISM 310 genetic analyzer (Perkin-Elmer). The alleles were numbered, and representative samples were sequenced with ABI PRISM 310 in both orientations to confirm the GeneScan results.

Statistical Analysis

The frequency distribution of the sociodemographic characteristics, including age, sex, cigarette smoking, and alcohol drinking, was examined for all subjects. Cigarette smoking status was categorized into never smoked and ever smoked. Those considered to have ever smoked were those who had smoked more than 100 cigarettes in their lifetime. The association between the *iNOS* (CCTTT)n promoter polymorphism and the pathologic and clinical characteristics (grade and stage) was assessed using the chi-square test. The effect of the *iNOS* (CCTTT)n promoter polymorphism on UC risk was calculated with the odds ratio (OR) and 95% confidence interval (CI), which was derived from unconditional multivariate logistic regression analysis. The Statistical Analysis Systems, version 6.12, software (SAS Institute, Cary, NC) was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

RESULTS

No significant differences were found in the distribution of age or sex between those with UC and the controls. After adjustment for age, sex, and alcohol drinking, a significantly increased UC risk of 2.3 and 2.4 was found for those who had ever smoked and those who had smoked more than 30 years, respectively. Regarding alcohol drinking, a significantly increased UC risk was found for those who had ever consumed alcohol, after adjustment for age, sex, and cigarette smoking (Table 1). The distribution of the polymorphic (CCTTT)n repeats is shown in Figure 1; the repeats range from 6 to 21. Among them, four repeats (10, 11, 12, and 13) with a greater frequency (more than 10%) and five repeats (6, 8 19, 20, and 21) with a rare frequency (less than 1%) were observed. Additionally, three longer repeat lengths were found in a greater proportion of the controls, including the 15-repeat allele (5.2% in patients with UC and 6.0% in controls; chi-square test, $P = 0.5882$), 16-repeat allele (4.6% in patients with UC and 6.4% in controls; chi-square test, $P = 0.2119$), and 17-repeat allele (3.0% in patients with UC and 3.6% in controls; chi-square test, $P = 0.5954$). However, we found nonsignificant differences between those with UC and controls among these three repeats. We then excluded the five rare repeats and defined alleles of less than 12 repeats as the short form (S) and alleles of 12 or more repeats as the long form (L). The study subjects were classified into three genotypes: S/S, S/L, and L/L. After we had combined the S/L and L/L genotypes, a nonsignificantly increased UC risk was shown (Table 1). However, the study subjects carrying the 12-repeat allele had a significantly increased UC risk. The patients with UC were then categorized into two groups (superficial and invasive) and a significant association was found with the *iNOS* (CCTTT)n promoter

Table 1. Risk of urothelial cancer by sociodemographic characteristics and *iNOS* (CCTTT)n promoter polymorphism among patients with UC and controls

Variable	Patients with UC (n = 250)	Controls (n = 250)	OR (95% CI)
Age (yr)			
<55	57 (22.8)	72 (28.8)	1.0*
55–69	89 (35.6)	96 (38.4)	1.1 (0.7–1.7)
≥70	104 (41.6)	82 (32.8)	1.3 (0.8–2.1)
Sex			
Female	96 (38.4)	83 (33.2)	1.0*
Male	154 (61.6)	167 (66.8)	0.9 (0.6–1.3)
Cigarette smoking			
Never	128 (51.2)	155 (62.0)	1.0†
Ever	122 (48.8)	95 (38.0)	2.3 (1.4–3.8)‡
Duration (yr)			
0	128 (51.2)	155 (62.0)	1.0†§
1–29	37 (14.8)	36 (14.4)	2.0 (1.1–3.8)¶
≥30	85 (34.0)	59 (23.6)	2.4 (1.5–4.2)‡
Alcohol drinking			
Never	195 (78.0)	218 (87.2)	1.0‖
Ever	55 (22.0)	32 (12.8)	2.0 (1.3–3.4)#
iNOS (CCTTT)n genotypes			
S/S**	45 (18.4)	56 (22.9)	1.0††
S/L	113 (46.1)	110 (44.9)	1.3 (0.8–2.1)
L/L	87 (35.5)	79 (32.2)	1.4 (0.9–2.4)
S/L + L/L	200 (81.6)	189 (77.1)	1.3 (0.9–2.1)
12-Repeat allele			
Noncarriers	157 (62.8)	178 (71.2)	1.0††
Carriers	93 (37.2)	72 (28.8)	1.7 (1.1–2.5)¶

iNOS = inducible nitric oxide synthase; UC = urothelial cancer; OR = odds ratio; CI = confidence interval; S = short; L = long.
* Adjusted for age and sex.
† Adjusted for age, sex, and alcohol drinking.
‡ $P < 0.001$.
§ P for trend < 0.001.
¶ $P < 0.05$.
‖ Adjusted for age, sex, and cigarette smoking.
** Five "rare" repeat numbers (frequency <1%) were excluded; other repeat numbers divided into two groups: S (9–11 repeats) and L (12–18 repeats).
†† Adjusted for age, sex, cigarette smoking, and alcohol drinking.

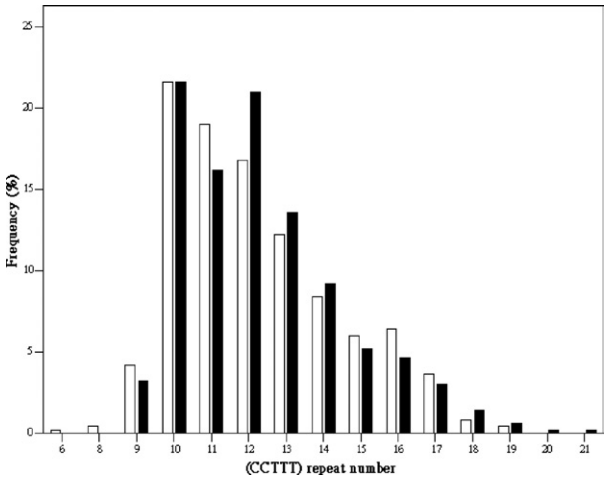

Figure 1. Distribution of (CCTTT)n promoter polymorphism in patients with UC (black bars) and controls (white bars).

polymorphism (S/S, S/L, and L/L; $P = 0.043$). However, we found no significant association between the pathologic grade and the investigated polymorphism (Table 2). The UC risk related to the (CCTTT)n promoter polymorphism was further examined in those with diverse cigarette smoking status (Table 3). After adjustment for age, sex, and alcohol drinking, those who had ever smoked had a significantly increased UC risk of 3.5 and 4.5 for those with the S/L and L/L genotypes and the 12-repeat allele, respectively. In addition, those who had smoked more than 30 years had a significantly increased UC risk of 2.4 and 3.8 for those with the S/L and L/L genotypes and the 12-repeat allele, respectively.

COMMENT

In this study, we observed that the polymorphic (CCTTT)n repeat in the *iNOS* promoter and cigarette smoking were significantly associated with UC risk. Consistent with the findings of previous studies,[21,22] cigarette smoking was the major risk factor for bladder cancer. We expected such a finding because cigarettes contain many carcinogens that put smokers at an increased risk of UC. We also found a significantly increased UC risk in alcohol drinkers. A meta-analysis suggested a slightly increased cigarette smoking-adjusted UC risk for men (relative risk 1.3).[22] Minor differences could have resulted from the different drinking patterns of various populations.

This is the first study to explore an association between

Table 2. Associations of *iNOS* (CCTTT)n promoter polymorphism with pathologic grade and clinical stage

Variable	S/S*	S/L	L/L	*P* Value†	12-Repeat Allele		
					Noncarriers	Carriers	*P* Value†
Grade				0.541			0.673
Low (G1)	16 (21.6)	35 (47.3)	23 (31.1)		45 (60.8)	29 (39.2)	
High (G2–G3)	29 (17.0)	78 (45.6)	64 (37.4)		112 (63.6)	64 (36.4)	
Stage				0.043			0.240
Superficial (≤T1)	33 (23.7)	61 (43.9)	45 (32.4)		93 (66.0)	48 (34.0)	
Invasive (T2–T4)	12 (11.3)	52 (49.1)	42 (39.6)		64 (58.7)	45 (41.3)	

Abbreviations as in Table 1.
Data in parentheses are percentages.
* Five "rare" repeat numbers (frequency <1%) were excluded; other repeat numbers divided into two groups: S (9–11 repeats) and L (12–18 repeats).
† Chi-square test.

Table 3. Joint effect on risk of urothelial cancer between *iNOS* (CCTTT)n promoter polymorphism and cigarette smoking among UC cases and controls

Variable	(CCTTT)n Genotypes	UC (n = 250)	Controls (n = 250)	OR* (95% CI)
Cigarette smoking				
Never	S/S†	22 (9.0)	36 (14.7)	1.0‡
	S/L + L/L	103 (42.0)	115 (46.9)	1.7 (0.9–3.1)
Ever	S/S	23 (9.4)	20 (8.2)	3.3 (1.4–8.3)§
	S/L + L/L	97 (39.6)	74 (30.2)	3.5 (1.7–7.3)¶
Duration (yr)				
<30	S/S	33 (13.5)	42 (17.1)	1.0‡
	S/L + L/L	129 (52.7)	145 (59.2)	1.2 (0.7–2.1)
≥30	S/S	12 (4.9)	14 (5.7)	1.5 (0.6–3.9)
	S/L + L/L	71 (28.9)	44 (18.0)	2.4 (1.3–4.7)§
Cigarette smoking	12-Repeat allele			
Never	Noncarriers	80 (32.0)	104 (41.6)	1.0‡
	Carriers	48 (19.2)	51 (20.4)	1.4 (0.8–2.3)
Ever	Noncarriers	77 (30.8)	74 (29.6)	2.1 (1.2–3.7)§
	Carriers	45 (18.0)	21 (8.4)	4.5 (2.2–8.9)¶
Duration (yr)				
<30	Noncarriers	106 (42.4)	132 (52.8)	1.0‡
	Carriers	59 (23.6)	59 (23.6)	1.3 (0.8–2.1)
≥30	Noncarriers	51 (20.4)	46 (18.4)	1.6 (0.9–2.7)
	Carriers	34 (13.6)	13 (5.2)	3.8 (1.8–8.0)¶

Abbreviations as in Table 1.
* Adjusted for age, sex and alcohol drinking.
† Five "rare" repeat numbers (frequency <1%) were excluded; other repeat numbers divided into two groups: S (9–11 repeats) and L (12–18 repeats).
‡ *P* for trend <0.001.
§ *P* <0.01.
¶ *P* <0.001.

the polymorphic (CCTTT)n repeat in the *iNOS* promoter and UC risk. The distribution of the (CCTTT)n repeats was similar to that reported previously for the Japanese and was different from that of other populations.[23] In our study, the repeats were spread between 6 and 21 in those with UC and 6 and 19 in the controls. Several reports have divided the length into short and long forms, depending on their ability to elevate iNOS expression. We defined alleles of 9 to 11 repeats as the short form (S) and alleles of 12 to 18 repeats as the long form (L). An in vitro study showed that *iNOS* promoter activity increases with the repeat numbers (9 to 15 repeats), suggesting that longer forms have greater transcription activity.[24] However, no significantly increased UC risk was found in our study subjects carrying the S/L and L/L genotypes. It is likely that different repeats have diverse effects on the transcription ability of the promoter. Therefore, the transcription effect was not solely dependent on the promoter length because the longer forms (15 and 17 repeats) were no more effective than the 14 repeat in mediating interleukin-1beta induction of iNOS.[24] In addition, we found a significantly increased UC risk in those carrying the 12-repeat allele. A previous study indicated that the frequent repeat was the 12-repeat allele and that 41% of the patients with colorectal cancer and 38% of controls carried this allele.[25] Moreover, a significantly greater frequency of the 12-repeat allele was observed in Chinese patients with diabetes compared with white patients with diabetes ($P = 0.001$).[26] Although the association between the 12-repeat allele and UC risk found in our study is not evidence of causation, we cannot exclude the possibility that the 12-repeat

allele is in linkage with the primary associated polymorphism.

Although patients with UC at an advanced stage (T2–T4) seemed to have a greater percentage of a longer *iNOS* (CCTTT)n promoter length, we have merely shown an association between stage and polymorphisms. However, previous studies have indicated that iNOS expression in bladder cancer tissue might play an important role in tumor angiogenesis and recurrence.[16,17] Because of the lack of follow-up of the patients with UC, we can only speculate whether the polymorphisms in the *iNOS* gene will lead to varying iNOS expression and alter the individual's susceptibility to UC. Furthermore, our results have indicated that of those who have ever smoked and those who have smoked more than 30 years, the individuals with S/L and L/L genotypes and the 12-repeat allele have significantly increased UC risks, respectively. One study has suggested that cigarette smoking results in oxidative stress, which leads to the stimulation of iNOS, together with the protein tyrosine phosphorylation, promoting the development of UC.[27]

Bladder instillation with BCG induces a local production of NO, likely because of the induction of NOS activity in urothelial cells.[28,29] To avoid the confounding from BCG instillations, those with recurrent UC and those who had received intravesical BCG instillations, radiotherapy, or chemotherapy preoperatively were excluded. In addition, the results of a recent study have refuted the correlation between urine or serum levels of NO and bladder cancer risk.[30] However, it had some limitations, including a small sample size, absence of the evaluation of healthy controls, and irregular and insufficient follow-up in the patients with cancer. Therefore, additional studies with longer follow-up and a larger number of patients with UC, controlling for treatment variation, are necessary to evaluate the true correlation between polymorphisms in the *iNOS* gene and UC risk.

CONCLUSIONS

This is the first study, to our knowledge, on the association among the (CCTTT)n repeat of *iNOS* promoter, cigarette smoking and UC risk. Our findings have suggested that the polymorphic (CCTTT)n repeat in the *iNOS* promoter might be involved in the development of UC, especially in those who have ever smoked.

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