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Androgen receptor gene polymorphism may affect the risk of urothelial carcinoma

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Abstract

The study sought to explore if androgen receptor gene (*AR*) polymorphisms are associated with the risk of urothelial carcinoma (UC) which is male-predominant. *AR* CAG and GGN repeat lengths were analyzed in 277 UC cases and 280 age and sex-matched controls by direct sequencing of leukocyte DNA. Smoking habits were obtained using a structured questionnaire interview. Relative risks were compared between groups categorized by all possible cutoffs of *AR* CAG and GGN repeat lengths. Men and women who had 23 and 44 (cumulative) CAG repeats had a significantly greater risk of UC, respectively (OR 2.09, 95% CI: 1.05–4.17, $p = 0.036$ and OR 4.95, 95% CI: 1.56–15.73, $p = 0.007$). Amongst males who were medium-dose cigarette smokers, those who had 23 CAG and shorter GGN (< 22) repeats, had an elevated risk than those with longer CAG and GGN (OR 4.32 and 4.57, $p = 0.034$ and 0.042, respectively). However, neither CAG nor GGN affected the UC risk in non-smokers or heavy smokers (≥25 packs per day-years). *AR* CAG polymorphism may affect the risk of UC in both genders. In addition, *AR* polymorphisms may influence carcinogenic effect of medium-dose of cigarette smoking in men.

Introduction

The androgen receptor (*AR*) gene is associated with numerous hormone-related cellular functions. The trans-activation activity of the receptor contains two polymorphic sites including a glutamine repeat encoded by CAG_nCAA and a glycine repeat encoded by $GGT_3GGG_1GGT_2GGC_n$, which are commonly referred to as the CAG and GGN repeats, respectively [1]. The lengths of the CAG and GGN stretches range from 8–35 to 10–35 repeats, respectively. It has been shown that the shorter the repeat length, the higher the expression of AR protein (GGN) or the trans-activation activity (CAG) [2, 3]. Several epidemiological studies have shown that CAG polymorphism is related to the risk of developing various human cancers, including gynecological and steroid hormone-related tumors, such as prostate [4], breast [5] and ovarian cancers [6]. In addition, a possible

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influence of the GGN repeats on the progression of prostate cancer has also been found [7].

In general, the male incidence of urothelial carcinoma (UC) is approximately 2–3 times that for females [8]. Hormonal differences are considered as a potential factor accounting for the sex disparity. Animal studies have demonstrated that the incidence of spontaneous and chemically induced bladder tumor development is significantly greater in male, than in female rats [9]. It has been found that invasive bladder tumors loose heterozygosity of the active allele of the X chromosome at the *AR* locus, suggesting that allelic loss of the activated X chromosome is involved in bladder tumorigenesis and cancer progression [10]. In addition, an immunohistochemical study provided evidence that invasive bladder cancer has a significantly reduced AR protein expression compared to that of tumors at earlier stages [11]. These studies support a role for androgens in urothelial tumorigenesis. Thus, sex hormone and X chromosome-linked genetic factors may be involved in urothelial tumorigenesis. However, to date there have been no reports that address the relationship between *AR* gene polymorphisms and the risk of UC.

A number of reports have demonstrated gene–environment interactions and an association with cancer susceptibility. It has been shown that the *AR* gene polymorphisms affect the risk of male predominant cancer, for example hepatitis B virus-related liver cancer [12]. The gender difference in UC may be partly attributed to exposure to various environmental risk factors, such as cigarette smoking, occupational and/or environmental carcinogens etc. The most well-documented environmental risk factor for UC is cigarette smoking and smokers have a two- to three-fold risk of developing bladder cancer compared to non-smokers [13].

Based on the above findings, we sought to explore whether the UC risk is affected by *AR* gene polymorphisms. Particular attention was focused on the interaction between *AR* gene polymorphisms and cigarette smoking.

Materials and methods

Study subjects

This is a case–control study. Participating subjects consisted of 277 cases of pathologically proven UC and 280 age (±3 years) and sex-matched controls who were free of any known malignancies. Cases with UC were accrued from the National Taiwan University Hospital (NTUH), Taipei, Taiwan. UC case subjects who had a second primary malignancy were excluded. Urothelial adenocarcinoma was also excluded. Of the 277 UC cases, 60, 162 and 55 patients had UC in the upper urinary tract (renal pelvis and ureter), lower urinary tract (bladder and urethra), and both, respectively. None of the cases or controls were from the UC endemic area in southwest Taiwan where chronic arsenic exposure from contaminated artesian well water has been shown to confer a significantly elevated risk of UC [14]. These subjects were excluded to avoid the overwhelming carcinogenic effect of arsenic exposure compared to the effects of smoking or *AR* gene polymorphisms.

Controls were accrued from two groups of subjects, including those who underwent general health checkups at the Taipei Medical University Hospital, Taipei, Taiwan (n = 79) and those who were hospital-based urological patients in NTUH (n = 201). The latter group included patients with benign prostatic hypertrophy (BPH), urolithiasis, voiding dysfunction, erectile dysfunction, urinary tract infection, etc. The study protocol was approved by the institutional review board (IRB) of all participating hospitals and all participants signed informed consents prior to enrollment into to the study.

Questionnaire interview

Well-trained personnel carried out a standardized personal interview based on a structured questionnaire. The history of cigarette smoking and possible environmental exposure were taken in detail. Smoking was defined as doing so at least three times a week for 6 months or longer. Former smokers were defined as those who had quit smoking at least 6 months prior to the diagnosis for case or accrual for control subjects. The average consumption per day in packs and duration in years was recorded. Cumulative consumption was calculated by packs consumed per day (PPD)-years product method.

Genetic analyses

The buffy coat of 10 ml of whole blood (in EDTA) was isolated and stored at −80°C until DNA extraction. Genomic DNA was extracted by using

the Puregene Genomic DNA Purification Kit (Gentra Systems Inc., Minneapolis, MN) and was stored at −20°C until further analysis. DNA was sent for *AR* genotyping, with each batch containing cases and controls, blinded to the laboratory technicians. The genotyping was PCR-based with the primers being as follows: *AR* CAG forward primer 5′-CAGCGACTACCG CATCATCA-3′ and the reverse primer 5′-TCT GGGACGCAACCTCTCTC-3′; *AR* GGN forward primer 5′-GAGCTCCGGGACACTTG AAC-3′ and the reverse primer 5′-GGCGACATT TCTGGAAGGAA-3′. These primers were used for both DNA amplification and DNA sequencing. DNA amplification was performed by using a thermal cycler (GeneAmp PCR System 9600, Perkin Elmer, Foster, CA). Reactions were carried out in 25 µl total volume containing 100 ng genomic DNA, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 10 nmol dNTP, 4% DMSO, 20 pmol/primer, and 1 unit of *Taq* polymerase. After an initial denaturation at 94°C for 2 min, PCR was carried out for 30 cycles at 94°C for 20 s, 55°C (CAG) or 50°C (GGN) for 30 s, and 72°C for 50 s. Final extension was performed at 72°C for 7 min. The PCR products were analyzed by electrophoresis in 1.2% agarose gels.

DNA sequencing to determine CAG and GGN repeat numbers

PCR products were purified by using the PCR-M® Clean Up System (Viogene, Sunnyvale, CA). The number of trinucleotide repeats was analyzed by DNA direct sequencing. Fluorescence-based DNA sequencing was performed with the API Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Foster, CA). All reactions were optimized to reach consistent results. For the polyglutamine tract (CAG_nCAA), the number of CAG triplets was counted to yield the length of CAG repeats. The GGN repeat number was counted according to the usual sense codon sequence of the GGN tract that includes the whole GGN stretch ($GGT_3GGG_1GGT_2GGC_n$).

Statistical analysis

The lengths of the CAG and GGN repeats were compared between cases and controls using several methods, as described previously [15, 16]. We compared the repeat numbers dichotomously or in tertiles with all possible cutoffs. Only the cutoffs or analyses that yielded the greatest difference amongst groups are shown. Since females have two alleles of the *AR* gene, while males have only one, analyses were done separately. For females, two methods of analysis were used. The cumulative repeat length was generated by adding the length of both alleles [15]. Alternatively, only the shorter or the longer allele length was compared between groups [16]. The Student's *t*-test was used to compare continuous variables between groups. Continuous variables are expressed as mean ± standard deviation where applicable. Univariate logistic regression was performed to compare smoking history and repeat length frequencies between groups. Multivariate logistic regression analysis was used to calculate odds ratios (OR) and 95% confidence interval (CI) after adjustment for age and cumulative smoking amount in PPD-years. All *p* values were two-sided.

Results

Demographics

Cases and controls did not differ significantly in sex or age distribution (Table 1). Since only four female subjects (< 4%), in either case or control group were current or former smokers, smoking analysis was done only for males. For males, 62.4% of the cases and 54.7% of the controls were current or former smokers. The relative risk of UC in men who smoked ≥25 PPD-years over non-smokers was 2.2-fold (95% CI: 1.33–3.72, $p = 0.002$).

CAG and GGN repeats versus UC risk

The mean CAG and GGN repeat lengths for males were 23.1 (range 12–34) and 22.5 (range 12–25), respectively (Figure 1A). Among females, the figures were 22.3 (range 13–32) and 22.2 (range 17–26), respectively, while the mean cumulative CAG and GGN repeat lengths were 44.6 (range 30–62) and 44.5 (range 37–51), respectively (Figure 1B). There were no significant differences in the mean CAG or GGN repeat numbers between cases and controls for either gender.

Table 1. Demographics, smoking history, and AR trinucleotide repeat numbers among urothelial carcinoma patients and matched controls.

	Cases *n* (%)	Controls *n* (%)	*p* value
Gender			
Female	105 (37.9)	108 (38.6)	
Male	172 (62.1)	172 (61.4)	
Total	277 (100.0)	280 (100.0)	
Mean age in years (SD)[a]			
Female	63.6 (13.6)	60.2 (18.1)	0.12
Male	62.5 (13.2)	61.8 (13.5)	0.62
Cumulative smoking amount in males (PPD-years)[b]			
0	65	81	
1–24	34	51	0.49[c]
≥25	70	40	0.002[d]
CAG repeat number[e]			
Male	17–31	12–34	0.77
Female (shorter)	13–30	14–30	0.51
Female (Longer)	17–30	18–32	0.69
Female (cumulative)	30–60	34–62	0.90
GGN repeat number			
Male	12–25	18–25	0.26
Female (shorter)	17–23	18–25	0.27
Female (Longer)	19–25	19–26	0.75
Female (cumulative)	38–48	37–51	0.34

[a]Student's *t*-test.
[b]Adjusted for age; by logistic regression; Smoking amount of three case subjects were not available; PPD-years: the product of packs consumed per day times years of consumption.
[c]Odds ratio: 0.82 (95% CI: 0.48–1.42).
[d]Odds ratio: 2.22 (95% CI: 1.33–3.72).
[e]Wilcoxon Rank-Sum test.

When CAG repeat lengths were compared in tertiles (Table 2), men with exactly 23 repeats had a significantly higher risk of UC than those with either >23 or <23 repeats, after adjustment for age and cumulative smoking amount (OR 2.09, 95% CI: 1.05–4.17, $p = 0.036$). There was no difference between groups of CAG repeats >23 and <23 in males. Similarly, women who had a cumulative CAG repeat lengths of 44 had a significantly higher risk than those with either >44 or <44 repeats (OR 4.95, 95% CI: 1.56–15.73, $p = 0.007$). There was also no difference in the risk between groups of CAG >44 and <44 repeats. When only the shorter or longer alleles were compared in females, no differences in the risk of UC were found between the tertile groups of CAG repeat length polymorphisms (data not shown). In addition, there were no differences in the risk of UC between dichotomous groups of CAG or GGN repeat polymorphisms for either sex (data not shown).

For GGN, no significant relationship was found between repeat length polymorphisms and the risk of UC in either sex as determined by dichotomous or tertile grouping.

There were two groups of control subjects in the study, including subjects from general health checkups and hospital-based urological patients with benign diseases. Since the pathogenesis of BPH may be related to the androgenic effect and *AR* CAG and GGN repeat polymorphisms may differ between BPH and non-BPH male subjects [17], the proportion of control subjects with BPH may affect the results. We did an additional analysis to examine the relationship between BPH and *AR* gene polymorphisms. Among the 172 male control subjects, 84 (48.8%) were symptomatic BPH patients. There were no significant differences in CAG or GGN repeat lengths between BPH and non-BPH male control subjects ($p = 0.17$ and 0.65, respectively).

Combined analysis of the CAG and GGN repeat length polymorphisms

We jointly analyzed the CAG and GGN polymorphisms to examine the combined effects on the risk of UC. Men who had 23 CAG repeats and ≥23 GGN repeats had a higher risk of UC than those with non-23 CAG repeats and a GGN repeat <23 (adjusted OR: 2.49, 95% CI: 1.06–5.85, $p = 0.037$, Table 3). Similarly, females who had cumulative 44 CAG repeats, and a cumulative GGN repeat of 44 or longer had a significantly higher risk than those who had non-44 CAG repeats and a GGN of 44 or longer (adjusted OR: 5.88, 95% CI: 1.61–21.50, $p = 0.007$, Table 3). The OR for men and women who had 23 and 44 (cumulative) CAG repeats were lower (OR 2.09 and OR 4.95, respectively, Table 2), when GGN repeats were not considered, indicating that although GGN alone did not significantly affect the risk, it may still play a minor role.

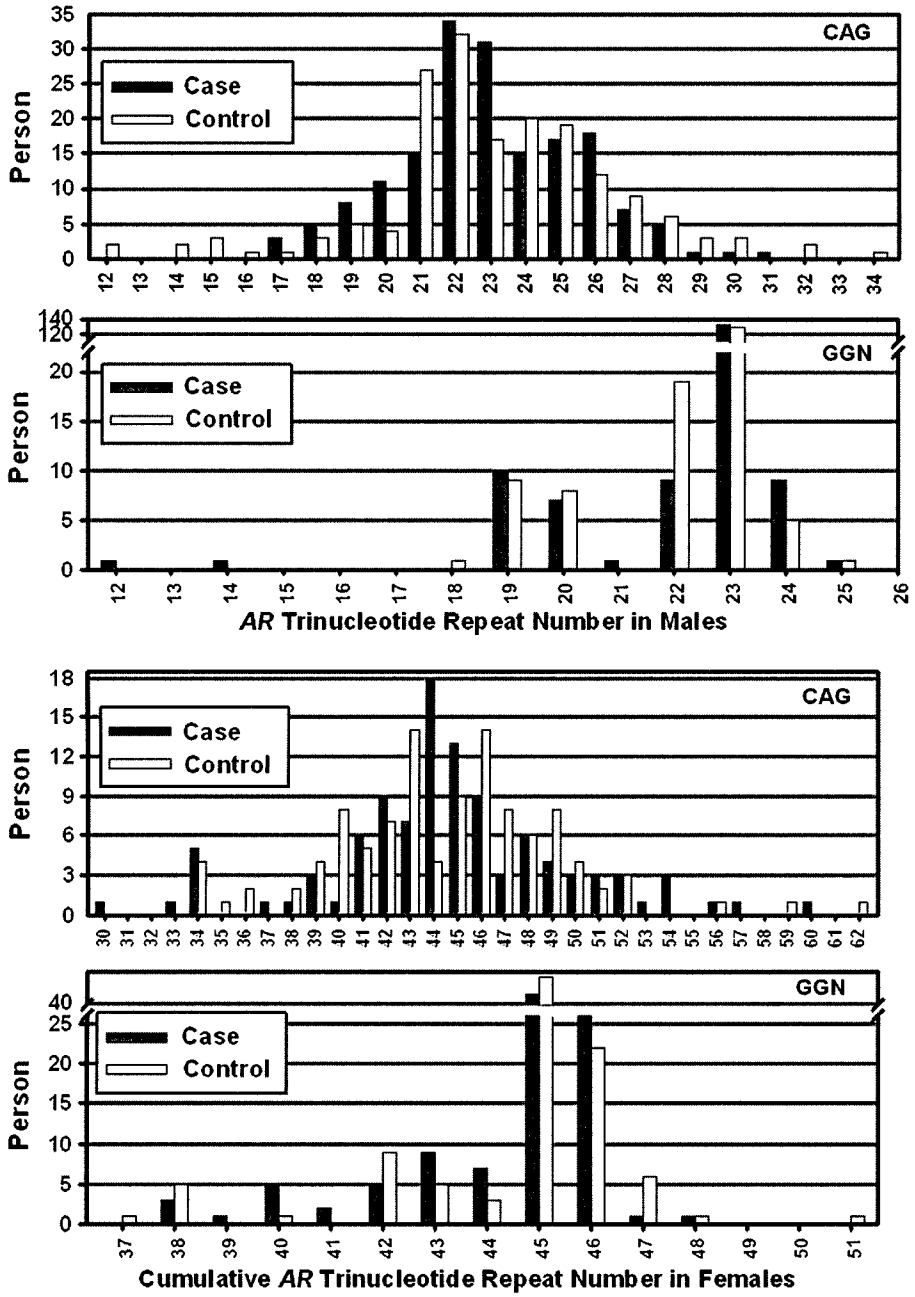

Figure 1. The frequency distribution of *AR* polymorphisms (CAG and GGN) in males (A) and females (B).

Smoking effects modified by AR gene polymorphisms

Male smokers who had 23 CAG repeats had a significantly increased risk of UC than those with a longer repeat, if they were medium smokers (1–24 PPD-years cumulatively, $p = 0.034$, OR 4.34, 95% CI: 1.12–16.89; Table 4). Male smokers who had a shorter GGN repeats (<22) had an increased risk than those with a longer repeat (OR 4.57, 95% CI: 1.06–19.79, $p = 0.042$), if they were medium smokers. However, neither CAG nor GGN polymorphisms affected the risks of never smokers or heavy smokers (≥25 PPD-years).

Table 2. Association of CAG repeat length polymorphism and risk of urothelial carcinoma.

CAG repeat length[a]	No. cases (%)	No. controls (%)	Crude odds ratio (95% CI) *p* value	Adjusted[b] odds ratio (95% CI) *p* value
Male				
CAG > 23	65 (37.8)	75 (43.6)	1.00	1.00
CAG = 23	31 (18.0)	17 (9.9)	2.07 (1.05–4.09)	2.09 (1.05–4.17)
			$p = 0.037$	$p = 0.036$
CAG < 23	76 (44.2)	80 (46.5)	1.10 (0.69–1.74)	1.09 (0.68–1.73)
			$p = 0.69$	$p = 0.73$
Female cumulative length[c]				
CAG > 44	51 (48.6)	57 (52.8)	1.00	1.00
CAG = 44	18 (17.1)	4 (3.7)	5.03 (1.60–15.84)	4.95 (1.56–15.73)
			$p = 0.006$	$p = 0.007$
CAG < 44	36 (34.3)	47 (43.5)	0.86 (0.48–1.52)	0.77 (0.42–1.39)

[a]Cutoffs shown here represent the ones that generate the largest differences between groups.
[b]Adjusted for age and cumulative smoking amount (PPD-years); by logistic regression.
[c]Sum of the repeat numbers of the two CAG alleles for females.

Discussion

To our knowledge, this is the first study exploring the relationship between *AR* gene polymorphisms and the risk of UC. Our data support the hypothesis that *AR* CAG repeat length polymorphism is associated with the risk of UC in both genders. The molecular mechanisms underlying the association are far from clear. It has been shown that the CAG stretch is able to form hairpin structures [18], which could affect the AR mRNA degradation susceptibility or the efficiency of movement along the ribosome to affect translation efficiency [19]. Thus, specific CAG repeat length may interact more effectively with specific RNA binding proteins, conferring a unique level of AR trans-activation function, and hence affecting urothelial tumorigenesis. Furthermore, more than 70 proteins are known

Table 3. Combined analysis of the CAG and GGN repeat lengths polymorphism and risk of urothelial carcinoma.

Trinucleotide groups[a]	No. cases (%)	No. controls (%)	Crude odds ratio (95% CI) *p* value	Adjusted[b] odds ratio (95% CI) *p* value
Male				
CAG ≠ 23, GGN < 23	23 (13.6)	34 (19.8)	1.00	1.00
CAG ≠ 23, GGN ≥23	116 (68.6)	121 (70.3)	1.42 (0.79–2.55)	1.38 (0.76–2.51)
			$p = 0.24$	$p = 0.29$
CAG = 23, GGN < 23	6 (3.6)	3 (1.7)	2.96 (0.67–13.03)	3.13 (0.70–13.91)
			$p = 0.15$	$p = 0.13$
CAG = 23, GGN ≥23	24 (14.2)	14 (8.1)	2.53 (1.09–5.90)	2.49 (1.06–5.85)
			$p = 0.031$	$p = 0.037$
Female cumulative length[c]				
CAG ≠ 44, GGN ≥44	66 (62.9)	84 (77.8)	1.00	1.00
CAG ≠ 44, GGN < 44	21 (20.0)	20 (18.5)	1.34 (0.67–2.67)	1.30 (0.65–2.61)
			$p = 0.41$	$p = 0.46$
CAG = 44, GGN ≥44	14 (13.3)	3 (2.8)	5.94 (1.64–21.53)	5.88 (1.61–21.50)
			$p = 0.007$	$p = 0.007$
CAG = 44, GGN < 44	4 (3.8)	1 (0.9)	5.09 (0.56–46.63)	5.74 (0.61–53.70)
			$p = 0.15$	$p = 0.12$

[a]Cutoffs shown here represent the ones that generate the largest differences between groups.
[b]Adjusted for age and cumulative smoking amount (PPD-years); by logistic regression.
[c]Only the length of the cumulative length was shown and compared.

Table 4. Modification of UC risk from cigarette smoking by AR gene polymorphisms in male subjects.

Cumulative smoking	CAG repeat			GGN repeat[a]	
	>23	=23	<23	≥22	<22
Never smokers					
Case	25 (40.3%)	11 (57.9%)	29 (44.6%)	61 (46.2%)	4 (28.6%)
Control	37 (59.7%)	8 (42.1%)	36 (55.4%)	71 (53.8%)	10 (71.4%)
Odds ratio (95% CI)	1.00	2.04 (0.72–5.78)	1.20 (0.59–2.42)	1.00	0.47 (0.14–1.56)
		$p = 0.18$	$p = 0.62$	$p = 0.22$	
1–24 PPD-years					
Case	9 (27.3%)	8 (61.5%)	17 (43.6%)	27 (36.0%)	7 (70.0%)
Control	24 (72.7%)	5 (38.5%)	22 (56.4%)	48 (64.0%)	3 (30.0%)
Odds ratio (95% CI)	1.00	4.34 (1.12–16.89)	2.12 (0.78–5.77)	1.00	4.57 (1.06–19.79)
		$p = 0.034$	$p = 0.14$	$p = 0.042$	
≥25 PPD-years					
Case	30 (68.2%)	11 (73.3%)	29 (56.9%)	61 (63.5%)	9 (64.3%)
Control	14 (31.8%)	4 (26.7%)	22 (43.1%)	35 (36.5%)	5 (35.7%)
Odds ratio (95% CI)	1.00	1.34 (0.36–4.98)	0.59 (0.25–1.38)	1.00	0.98 (0.30–3.18)
		$p = 0.67$	$p = 0.23$	$p = 0.97$	

[a]Cutoff shown here is mean GGN repeat length for males.
Adjusted for age; by logistic regression.

to interact with the AR protein [20–22]. The CAG repeat region is located in the domain that is known to interact with some AR co-regulators [23]. Thus, the specific 23 CAG repeats may interact with the AR co-regulator milieu. It will be important to investigate the co-regulator milieu of AR polymorphic proteins regarding their trans-activation activity in tumorigenesis.

We also showed that the 23 CAG repeats might enhance the carcinogenic effect of cigarette smoking, when the cumulative consumption is modest (1–24 PPD-years). The reason why 23 CAG repeats do not increase the UC risk in heavy smokers may be that heavy smoking conveys an overwhelming carcinogenic effect, which surpasses the genetic effects exerted by *AR* gene polymorphisms. Only in smokers with modest consumption, where the carcinogenic effect is weak, and can additional minor risk factors such as a 23 CAG repeat play a role. It seems, however, that the potential synergistic carcinogenic effect of the two risk factors only works with susceptible subjects. Moreover, the finding that *AR* gene polymorphisms modify the smoking-related UC risk is further supported by the fact that not only 23 CAG repeats increased the risk ($p = 0.034$), but also short GGN was found to elevate the risk in modest smokers ($p = 0.042$). Although cigarette smoking has been known to be associated with an elevated risk of bladder cancer [24]. However, our data again provides evidence of a genetic predisposition and the effects of smoking which modifies the risk of UC.

There have been a number of studies addressing the interaction of other genetic factors and the effects smoking on the risk of UC [25–27]. Garcia-Closas et al. [25] investigated the polymorphisms of a number of genes and the risk of bladder cancer in a meta-analysis. They found that the *NAT2* slow-acetylator genotype increases the risk, especially amongst cigarette smokers. In a previous study, we have also shown that *NAT1* and *NAT2* may modulate the susceptibility to bladder cancer associated with cigarette smoking [28]. In addition, it was shown that the overall OR for hepatocellular carcinoma in Taiwanese men was 1.72 (95% CI: 1.03–2.89) for hepatitis B virus carriers with 20 or fewer *AR* CAG repeats, compared with those with more than 24 repeats [12, 29].

Little is known about the relationship between AR and smoking-related carcinogenic effects. There is, however, some indirect evidence that supports the existence of such a relationship. It has been shown that polymorphisms of both the *AR*

CAG repeat and the ornithine decarboxylase gene may impact on smoking effects and the risk of prostate cancer [30]. It has also been demonstrated that an F344 rat model of prostate cancer can be induced by exogenous testosterone plus 3,2′-dimethyl-4-aminobiphenyl [9, 31]. Of note, several studies have indicated that serum total or free testosterone levels can be increased by cigarette smoking [32, 33]. Also, higher testosterone levels can increase AR nuclear localization and trans-activation and thus increase cell proliferation [34]. Taken together, urothelial tumorigenesis may be enhanced by the interaction of cigarette smoking and a specific AR trans-activation function. However, more work is required to elucidate the mechanism of how smoke carcinogens affect the androgen-AR axis.

It has been shown that the X chromosome will be randomly inactivated by methylation in women [35]. Our findings do not rule out a possible impact of the CAG or GGN polymorphisms on X chromosome inactivation. Further methylation studies to determine allelic X chromosome inactivation or the AR function may be conducted to confirm the association between UC and *AR* CAG or GGN alleles in female.

Although the sample size of the entire study population (n = 557) is satisfying. Subgroup analyses were done based on a relatively small sample size, which may reduce the power of statistical results. Hence, further studies with specific endpoints of that kind are necessary to confirm the results.

In conclusions, our results suggest that *AR* CAG polymorphism may affect the risk of UC in both genders. In addition, *AR* polymorphisms may influence carcinogenic effects of modest cumulative levels of cigarette smoking in men.

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