CCND1 1722 polymorphism and potential relevance to upper tract urothelial cancer

HUI-HUI LIN[1,2], HUNG-LUNG KE[1,3], KUANG-HUNG HSIAO[1,2], RU-YIN TSAI[5], WEN-JENG WU[3,4], DA-TIAN BAU[5,6], LIN-LI CHANG[1,2]

[1] Department of Urology, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, ROC

[2] Graduate Institute of Medicine and [3]Department of Microbiology, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC

[4] Department of Urology, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC

[5] Terry Fox Cancer Research Laboratory, China Medical University Hospital, Taichung, Taiwan, ROC

[6] Graduate Institutes of Clinical Medical Science, China Medical University, Taichung, Taiwan, ROC

Corresponding to: Lin-Li Chang, Da-Tian Bau, Department of Microbiology, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 807, Taiwan

E-mail address: datian@mail.cmuh.org.tw; artbau1@yahoo.com.tw

Abstract. Background: The cell cycle regulator *cyclin D1 (CCND1)* is thought to play a major role in the transition of cell cycle from G1 to S phase. It is known that cancer cells have an unbalanced cell cycle regulation. However, the genetic role of *CCND1* in urothelial cancer was never known. Materials and Methods: This study was conducted to explore the association between the *CCND1* C1722G polymorphism and the susceptibility and progression of urothelial cancer. The *CCND1* genotypes of 170 patients and 249 control subjects were determined by PCR-RFLP and evaluated of their correlations with the clinical and histopathological data. Results: The genotypic results showed that *CCND1* GC or GC + CC genotypes was both more frequently observed in urothelial patients than the control individuals (P=0.05 and 0.03, respectively), and people carried GC genotype have 1.6-fold increased risk of urothelial cancer, compared with those carried GG genotype (P=0.05). Also, GC + CC genotype had 1.68-fold higher risk of urothelial cancer compared with GG genotype (P=0.03). In addition, *CCND1* genotype was significantly associated with ureter tumor (P=0.005) and advanced tumor status (P=0.019). Conclusion: At the meanwhile, no association between *CCND1* C1722G genotypes and tumor grade, survival and tumor recurrent was found. To sum up, our data suggested that the C allele of *CCND1* C1722G polymorphism may be a potential predictive and prognostic biomarker for advanced urothelial cancer, especially ureter tumor of upper tract urothelial cancer.

Key words: CCND1 1722, cyclin D1, polymorphism, upper tract urothelial cancer

Upper tract urothelial cancer (UC) is relatively rare in the West, where a ratio of 3:1:51 is reported for the incidence of UC of the renal pelvis, ureter and bladder, respectively (1). Due to the unusually high incidences of UC of the upper tract in Taiwan with a ratio of renal pelvis to ureter to bladder of about 1:2.08:6.72, it is valuable to study the specificity of Taiwan and then compare the counterpart findings in West populations. Increase incidence of upper tract UC may be associated with arsenic exposure, smoking, analgesics abuse, occupational carcinogens, hypertension, long standing urinary obstructions, infection and Balkan nephropathy (2-7). Recent study has provided evidence that genetic polymorphisms may also predispose to the development of cancer disease (8).

Cyclin D1 (CNND1) is a key regulator of G1-S cell cycle progression and overexpression of cyclin D1 is implicated in the etiology of several cancers including transitional cell carcinoma of the bladder (9-11). In addition, CNND1 was considered play an important role in early stage of urothelial tumorigenesis and has been shown to correlate with early recurrence, tumor differentiation and clinical outcome in bladder cancer (12, 13).

The gene *CCND1* is located on human chromosome 11q13. Polymorphism in *CCND1* with a common G to A substitution at nucleotide 870 in exon 4 of the gene has been described in 1995 (14). During these years, several studies showed the

CCND1 870 AA genotype had an increased risk and influences the outcome for several malignancies including bladder cancer (15-18). However, another G to C polymorphism at nucleotide 1722 within *CCND1* 3' untranslated region (3′ UTR) was seldom investigated (19). The first study reporting an association between *CCND1* 1722 polymorphism and cancer risk of squamous cell carcinoma of the head and neck was reported by Holley in 2001 (20). However, the influence of *CCND1* C1722G on tumorigenesis of other cancers was not reported.

Thus, his study was aimed at exploring the association between *CCND1* C1722G genotype and the susceptibility of UC, and the correlation of *CCND1* C1722G genotype with clinicopathological outcomes.

Materials and Methods

Study population and clinicopathological data collection. A total of 170 patients with Transitional cell carcinoma (TCC) of UC were recruited at Kaohsiung Medical University medical center between Jan 2006 to Dec 2007, all of whom were diagnosed with UC by pathologic examination of specimens obtained by biopsy or surgical resection. The clinical and histopathologic information and a cigarette smoking history were collected from patient charts and pathologic reports. The information was reviewed, and the data were entered into the database. The tumor

stage was assigned according to the TNM staging system (21), and the pathologic grade was determined according to the World Health Organization criteria (22). Two hundreds and forty nine 249 healthy individuals, who had been matched with the patients with age, admitted to the same hospital for health checkup and who had no previous diagnosis of urologic neoplastic disease or other malignancy were enrolled as controls. However, no information on smoking status was obtained in the control subjects. During the recruitment period, all the subjects enrolled were provided an informed consent and Human Research Committees of participating hospitals has approved this study. This study has also been reviewed by the Institutional Review Board (IRB) of Kaohsiung Medical University with the approval number of KMU-IRB-950195.

Genotyping conditions. Genomic DNA for analysis was extracted from blood specimens using proteinase K digestion following phenol-chloroform extraction as described previously (23). Genotyping for *CCND1* C1722G of all subjects was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The 159 bp fragments containing the polymorphic nucleotide were amplified using the forward primer 5'-CTCTTGGTTACAGTAGCGTAGC-3' and reverse primer 5'-ATCGTAGGAGTGGGACAGGT-3'. The following cycling

conditions were performed: 5 min of initial denaturation at 95^{o}C, 35 cycles of 30 sec of denaturation at 95^{o}C, 30 sec of annealing at 54^{o}C and 1 min of elongation at 72^{o}C; and 7 min of final extension at 72^{o}C. The PCR products were further digested with Hae III (New England, Biolabs, Beverly, MA), and then visualized by ethidium bromide stained 3% agarose gel electrophoresis with the help of UV light. On digestion with *Hae* III, the PCR product arising from the G allele was cut into fragments of 111, 26 and 22 bp, whereas C allele was cut into fragments of 137 and 22 bp (Fig 1). Sequences were confirmed by direct sequencing of 10% of the samples, and the results were 100% concordant.

Statistical analysis. To ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, the deviation of the genotype frequencies of *CCND1* single nucleotide polymorphisms in the control subjects from those expected under the Hardy-Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson's Chi-square test or Fisher's exact test (when the expected number in any cell was less than five) was used to compare the distribution of the *CCND1* genotypes between cases and controls. Cancer risk associated with the genotypes was estimated as odds ratio (ORs) and 95% confidence intervals (CIs) using unconditional logistic regression. Data was recognized as

significant when the statistical two-tailed *P*-value was less than 0.05.

Results

The characteristics of 170 patients and 249 control subjects were shown in Table I. The UC patients comprised 89 males and 81 females, and the control group comprised 140 males and 109 females. The mean age of the patients and control individuals was 66.8 and 60.1, respectively. No significant difference was found between the UC patient and the control groups in the distribution of gender or age ($P>0.05$) (Table I).

Genomic DNA obtained from patients and control groups were subjected to genotype analysis of the *CCND1* C1722G polymorphism, and the *CCND1* C1722G genotypes were presented in Table I. The rationale and electrophoregram of PCR-RFLP of *CCND1* C1722G were presented in Fig 1. Both allele distribution frequencies of the patient and the control groups fitted the Hardy Weinberg equilibrium. Compared with *CCND1* 1722 GG genotype, patients with the GC genotype tended to have 1.6-fold increased risk of UC ($P=0.05$; OR=1.602, 95%CI=0.996-2.578). Patients with GC + CC genotype had 1.68-fold increased risk of UC compared with individuals with the GG genotype ($P=0.03$; OR, 1.677, 95% CI, 1.046 to 2.687). It seemed that the C allele is a risky genetic factor for UC.

The 170 UC patients comprised subjects suffering from renal pelvic (29, 17.0%), ureter (28, 16.5%), bladder (93, 54.7%), and multi-site tumors (20, 11.8%).

The stratification analysis revealed that the *CCND1* C1722G genotypes had a significant association with ureter tumors, but not other tumors (P=0.005; Table II). Again, the C allele seemed to be risky genetic factor for ureter tumor of urothelial carcinoma. The association between *CCND1* C1722G genotypes and pathological state and clinical outcome were also examined in this study. Of the 170 UC patients, 22.9% were of organ-involved advanced tumors (≧pT3) and 60% were of high-grade (G3) tumors. Significant association between *CCND1* 1722 genotypes and advanced tumors was found (P=0.019; Table II). It indicated that different *CCND1* C1722G genotypes especially CC genotype may be associated with tumor aggressiveness. However, no statistically significant differences were found among different *CCND1* C1722G genotype with tumor grade (P=0.879), survival (P=0.648) and tumor recurrent (P=0.313) (Table II).

Since smoking habits were a well-known environmental factor for UC, we were also interested in the gene-environment interaction of *CCND1* C1722G genotype and smoking status. The results showed that no differential genetic distribution of *CCND1* C1722G genotypes between the smoking and nonsmoking groups (P=0.153, Table III). Furthermore, the association between *CCND1* C1722G genotype and the tumor grade, tumors stage, survival, recurrent was not influenced by smoking status (Table III).

Discussion

Cyclin D1 impinges on several distinct pathways that govern cancer cell proliferation. Although intragenic somatic mutation of cyclin D1 in human disease is rare, cyclin D1 gene translocation, amplification and/or overexpression are frequent events in selected tumor types. In literature, the polymorphism in the cyclin D1 locus that may affect splicing has been implicated in increased cancer risk or poor outcome was reported (17). Polymorphism in *CCND1* with a common G to A substitution at nucleotide 870 in the splice donor region of exon 4 of the gene has been shown to be related with a poor progression in several cancers including urothelial cancer (24, 25). Wang *et al* (18) indicated the possibility that the *CCND1* 870 AA genotype confers elevated risk of bladder cancer, with more pronounced risk among non-smoking cases and for bladder cancer of higher grade and stage. Ito *et al.* demonstrated the *CCND1* 870 AA genotype was associated with a 3.67-fold and 4.17-fold increased risk of the bladder cancer risk compared with GG and GA genotype, respectively (16). Ito's findings indicated that the *CCND1* 870 A allele may have a recessive effect on the genesis of cancer risk but not associated with the recurrence of urothelial cancer (16).

It has been known that *CCND1* 870 AA genotype will influence the alternatively spliced forms of the *CCND1* mRNA and produce variant transcript-b (14). The transcript-b may have a longer half-life since it lacks the PEST

(praline-serine-threonine)-rich region for rapid degradation (25), hence may alter the normal regulation of the cell cycle. Under such circumstances, the *CCND1* A allele could exert an effect on the aggressive behavior of the cancer cells. Our team has also conducted the association study of *CCND1* A870G, with a negative finding in ureter cancer (data not shown).

In this paper, we focused on another important polymorphic site of *CCND1,* the *CCND1* C1722G, which was rarely studied and discussed in cancer research in the literature. Among the limited reports, Holley's was very typical and worth of our notice, for this study was conducted to examine the significance of the *CCND1* 1722 polymorphism in bladder cancer (20). The results showed that the *CCND1* 1722 GC and CC genotype was more frequently observed in the bladder cancer group than the control group, suggesting that individuals with *CCND1* 1722 GC or CC genotype were increasing 1.68-fold risk of bladder cancer compared with the GG genotype. However, their sample size is limited with only 69 of the bladder cancer cases. We have extended the sample collection to all types of upper tract UC and the sample size was much larger (Table I). We found that the *CCND1* 1722 variant C allele was associated with an increased risk of ureter tumor, not bladder cancer or other types (Table II). Furthermore, the *CCND1* 1722 genotype especially CC was associated with advanced tumor, indicating an important influence of the *CCND1* 1722 genotype

on the tumor aggressiveness (Table III).

The most established risk factors for bladder cancer are occupational exposure to certain arylamines and exposure to cigarette smoke. Tabacco consumption has been shown to have a two to five folds higher risk than nonsmokers (24). Therefore, it is noteworthy to evaluate whether individuals have different *CCND1 C*1722G genotypes, when with tobacco consumption will increase UC risk. Although increasing risk of UC in individuals with *CCND1* 1722 CC genotype, no significant difference was found in subjects with or without a history of smoking. It indicated that *CCND1* 1722 genotypes influence the risk of suffering from UC, in spite of tobacco consumption.

High incidence of upper urinary tract urothelial carcinoma have been reported from the endemic area for “blackfoot disease” of Taiwan, and arsenic contaminated water was considered to be the reason for such high prevalence (26, 27). Nevertheless, water hygiene has improved in recent years, and yet the incidence of upper urinary tract urothelial carcinoma remains high; high incidence of upper urinary tract urothelial carcinoma also occurs in people who grow-up overseas, hence factors other than arsenic water contamination was suggested to contribute to the unusually high incidence. So far, no apparent explanation was found to account for this high prevalence of upper urinary tract urothelial carcinoma in Taiwan. The result of our study revealed that genetic polymorphism in *CCND1* C1722G may play an important

role in increasing risk of upper tract UC. However, it is still unclear whether etiologic effect of the *CCND1* polymorphism in the context of one or more additional environmental factors will cause high occurrence of upper tract UC in Taiwan. In addition, how *CCND1* C1722G polymorphism influences protein expression is still not clear since its location is in the 3'UTR.

In the situation that the cases are rare and not easy to collect within limited time period, we have enrolled as many as age- and gender-matched controls to strengthen the analyzing power of the case-control study. The lack of significance at borderlines from the analysis of both odds ratios and *P*-value here encourage us to confirm this preliminary finding in a larger case samples in the future, and also the studies in West countries are warranted. The limitation of case sample size temporarily did not restrict us for analysis of gene-environment interactions, such as the well-known risky smoking habit associated with ureter cancer (28-30). We have also examined the association of *CNND1* genotypes with important clinical indexes, such as cancer stages and grades.

In conclusion, this study suggested that *CCND1* 1722 CC genotype is associated with a higher risk of UC, especially associated with ureter cancer and aggressive tumor. This finding warrants individuals with GC or CC genotype need to pay attention in disease occurrence. Furthermore, C allele of *CCND1* C1722G may be

used as an accessory marker for susceptibility and disease progression of UC.

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Figure legends

Fig. 1 (a) Restriction map of *CCND1* 1722 genotypes. On digestion with *Hae* III, the PCR product arising from the G allele was cut into fragments of 111, 26 and 22 bp, whereas C allele was cut into fragments of 137 and 22 bp. (b) Electrophoregram of PCR-RFLP of *CCND1* 1722. Lane 1, 50 bp MW marker; Lane 2, 159 bp PCR product; Lane 3, GG homozygote; Lane 4, GC heterozygote; Lane 5, CC homozygote.

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Table I. Characteristics and *CCND1* C1722G genotypes among UC cases and healthy controls.

Characteristics	Cases (n=170)	Control (n=249)	OR (95% CI)	*P*-value
Sex				
Male	89	140		
Female	81	109		0.434
Mean age	66.8	60.1		
CCND1 C1722G				
GG	125	205	1.000 (Reference)	
GC	43	44	1.602 (0.996~2.578)	0.050
GC+CC	45	44	1.677 (1.046~2.687)	0.030

OR: odds ratio, 95% CI: 95% confidence interval

Table II. Characteristics of *CCND1* 1722 polymorphisms with tumor location, pathological grade and stage in patients with UC.

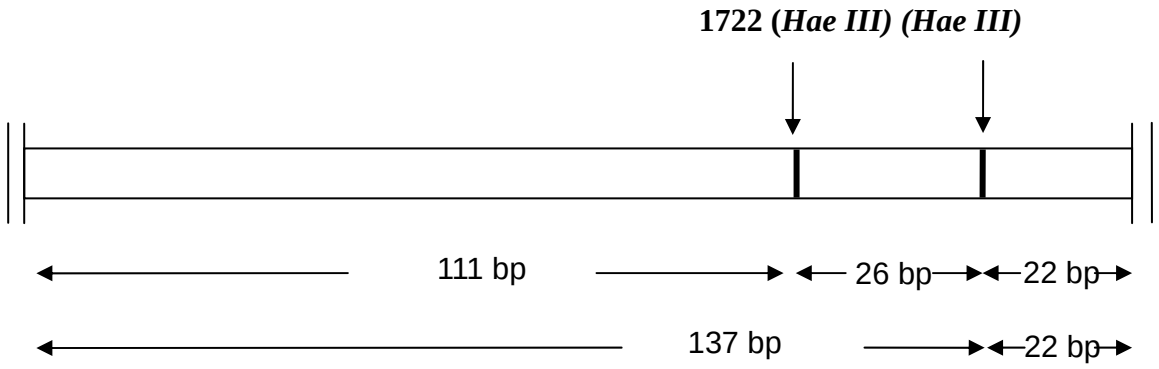
Genotypes	GG, n (%)	GC, n (%)	CC, n (%)	*P*-value
Location				0.047
Renal pelvic tumor	19 (15.2)	10 (23.3)	0 (0)	0.389
Ureter tumor	19 (15.2)	7 (16.3)	2 (100)	**0.005***
Bladder tumor	70 (56.0)	23 (53.5)	0 (0)	0.283
Multiple tumors	17 (13.6)	3 (7.0)	0 (0)	0.444
Grade				
Low	51 (40.8)	16 (37.2)	1 (50)	
High	74 (59.2)	27 (62.8)	1 (50)	0.879
Stage				
＜pT3	100 (80.0)	31 (72.1)	0 (0)	
≧pT3	25 (20.0)	12 (27.9)	2 (100)	0.019
Survival	118 (94.4)	39 (90.7)	2 (100)	0.648
Recurrent	54 (43.2)	13 (30.2)	1 (50.0)	0.313

* Statistically significant

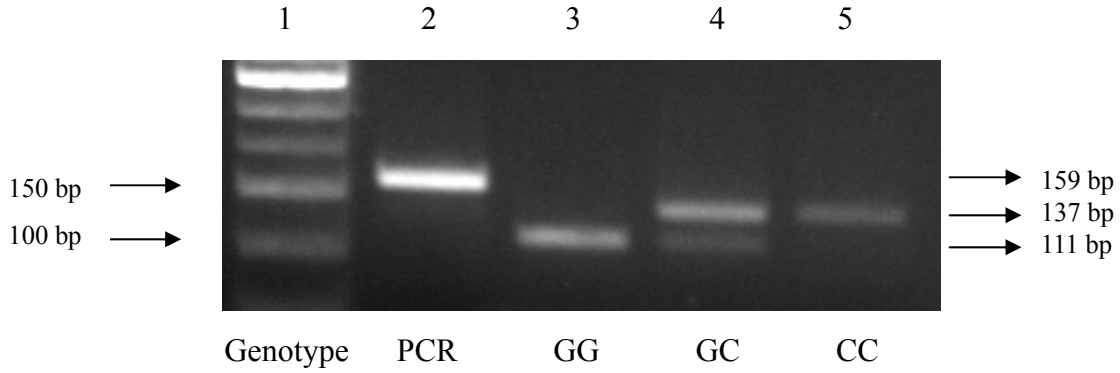
Table III. Risk of smoking on the tumor grade, tumors stage, survival, recurrent in patients with variant CCND1 1722 polymorphisms.

Genotypes	GG, n (%)	GC, n (%)	CC, n (%)	*P*-value
Smoking	38 (30.4)	19 (44.2)	0	0.153
Smoking+high grade	19 (50.0)	13 (68.4)	0	0.186
Smoking+≧pT3 tumor	8 (21.1)	4 (21.1)	0	1.000
Smoking+recurrent	20 (52.6)	7 (36.8)	0	0.260

(a)


1722 (*Hae III) (Hae III)*
111 bp
26 bp
22 bp
137 bp
22 bp

(b)


1
2
3
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150 bp
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137 bp
111 bp
Genotype
PCR
GG
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