# The potential role of *CCND1* G870A genotype as a predictor for urothelial carcinoma susceptibility and muscle-invasiveness in Taiwan

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**Running head:** Lin *et al*: *CCND1* Polymorphism in urothelial carcinoma

**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 a commonly *CCND1* G870A genotype was associated with bladder cancer in Japan and China, but not in Caucasian. There is neither report about its role in Taiwan population, nor the genetic role of *CCND1* G870A in another urothelial cancer, upper tract urothelial cancer (UTUC) worldwide. Therefore, we aimed at investigating its role in both bladder cancer and UTUC in Taiwan. Materials and Methods: The *CCND1* G870A genotypes of 171 (101 bladder cancer and 70 UTUC) patients and 243 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* GG genotype was associated with a lower risk in overall urothelial patients ($P$=0.008, OR=0.44, 95%CI=0.24-0.81) and bladder cancer patients ($P$=0.008, OR=0.34, 95%CI=0.15-0.76) than those of AA genotype. In addition, patients carried AG genotype have a 0.29-fold lower odds ratio of muscle-invasive cancer procession (95%CI=0.12-0.70), compared with those carried AA genotype in bladder cancer. But to our surprise, the GG genotype have a 5.88-fold higher odds ratio of muscle-invasive cancer procession (95%CI=1.08-32.01), compared with those carried AA genotype in UTUC. No association between any *CCND1* G870A genotype and higher grade risk was found. Conclusion: Our results

suggested that the G allele of *CCND1* G870A polymorphism may be a potential predictive and prognostic biomarker to distinguish bladder cancer and UTUC in Taiwan.

*Key words: CCND1* G870A, cyclin D1, polymorphism, urothelial carcinoma, bladder cancer, upper tract urothelial cancer

Cancers of the urinary system is among the most frequent malignancies all over the world. Among them, kidney and bladder cancers are more common worldwide. Renal cell carcinoma (RCC), accounts for the majority of the kidney neoplasms, and transitional cell carcinoma (TCC) of the bladder and upper tract is the fourth most frequent malignancy in male (1). In the West and in Taiwan, the incidence ratios for TCC of pelvis, ureter and bladder, were quite different. The former is 3:1:51, while the latter is 1:2.08:6.72 (2, 3). Upper tract urothelial cancer (UTUC) is relatively rare in the West and the unusually high incidences of UTUC in Taiwan makes it valuable to study the specificity of Taiwan and then compare the counterpart findings in West populations. In Taiwan, the increased incidence of UTUC may be associated with arsenic exposure, smoking, analgesics abuse, occupational carcinogens, hypertension, long standing urinary obstructions, infection and Balkan nephropathy (4-9). Recent study has provided evidence that genetic polymorphisms may also predispose to the development of cancer disease (10).

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 (11-13). 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 (14, 15). The gene *CCND1* is located on human chromosome 11q13. Polymorphism in *CCND1* with a common G to A substitution at nucleotide polymorphism G870A in exon 4 of the gene has been described in 1995 (16). During the recent years, several studies showed the *CCND1* 870 AA genotype had an increased risk and influences the outcome for bladder cancer (17-22). However, there is no literature investigating another type of urothelial cancer, UTUC.

Thus, this study was aimed at exploring the association between *CCND1* G870A genotype and the susceptibility of urothelial carcinoma, including both bladder and UTUC, and the correlation of *CCND1* G870A genotype with clinicopathological outcomes in Taiwan.


**Materials and Methods**

*Study population and clinicopathological data collection.* A total of 171 (101 bladder cancer and 70 UTUC) with TCC were recruited at Kaohsiung Medical University medical center between Jan 2006 to Dec 2007, all of whom were diagnosed with urothelial cancer 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 (23), and the pathologic grade was determined according to the World Health Organization criteria (24). Two hundred and forty-three 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 (25). Genotyping for *CCND1* G870A of all subjects was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay (26-28). The 167 bp fragments containing the polymorphic nucleotide were amplified using the forward primer 5'-GTGAAGTTCATTTCCAATCCGC-3' and reverse primer 5'-GGGACATCACCCTCACTTAC-3'. The following cycling conditions were

performed: 5 min of initial denaturation at 95$^{\circ}$C, 35 cycles of 30 sec of denaturation at

95$^{\circ}$C, 30 sec of annealing at 54$^{\circ}$C and 1 min of elongation at 72$^{\circ}$C; and 7 min of final

extension at 72$^{\circ}$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 *ScrF*I, the

PCR product arising from the A allele was uncut 167 bp, whereas G allele was cut

into fragments of 145 and 22 bps (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 genomic DNA obtained from 171 patients and 243 controls were subjected to genotype analysis of the *CCND1* G870A polymorphism, and the *CCND1* G870A genotypes were presented in Table I. The rationale and electrophoregram of PCR-RFLP of *CCND1* G870A were presented in Fig 1. Both allele distribution frequencies of the patient and the control groups fitted the Hardy Weinberg equilibrium. Compared with *CCND1* G870A AA genotype, patients with the GG genotype tended to have 0.44-fold risk of UC ($P$=0.008; OR=0.44, 95%CI=0.24-0.81). Patients with AG + GG genotype had also a significant 0.65-fold risk of UC compared with individuals with the AA genotype ($P$=0.039; OR=0.65, 95% CI=0.43-0.98). It seemed that the G allele is a protective genetic factor for UC. We have further divided the patients into bladder cancer and UTUC subgroups and reevaluated of their risk of UC. Interesting, bladder cancer patients with the GG genotype tended to have further lower risk of UC ($P$=0.008; OR=0.34, 95%CI=0.15-0.76) compared with those with AA type. But this trend is not observed in the UTUC patients (Table I).

The association of *CCND1* G870A genotypes with pathological characteristics in both bladder cancer and UTUC patients is presented in Table II. The first stratification parameter is the muscle-invasion issue. Clinically, the muscle-invasive and non-muscle invasive types of urothelial cancer, mainly determined by

pathological findings, may differ greatly in their etiology and clinical outcomes such as recurrence, progression, and patient survival. Take bladder cancer for instance, numerous factors are involved in the recurrence, progression, and patient survival rates environmentally and hereditarily (29). However, the genetic factors are largely unknown. Out data showed that compared with AA genotype, people of the AG genotype were of lower risk for muscle invasiveness ($P$=0.009; OR=0.29, 95% CI=0.12-0.70) in bladder cancer. On the contrary, people of the GG genotype were of higher risk for muscle invasiveness ($P$=0.039; OR=5.88, 95% CI=1.08-32.01) in UTUC, also compared with AA genotype (Table II). Second, patients with similar stage but different grades respond to treatment differently (30). Thus, all the patients of early stage were genotyped then further stratified by their pathological stages and analyzed of their risk. The results showed that in both bladder cancer and UTUC, neither of the genotype of *CCND1* G870A was positively associated with muscle-invasive risk (Table II).

**Discussion**

Cyclin D1 plays a critical role in the G1 to S transition phase of the cell cycle progression and is important for regulation of cell proliferation, differentiation, and transcriptional control (31). 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 (19). 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 urothelial cancer (32, 33). In this study, we hypothesized that the CCND1 G870A polymorphism may be associated with the risk of urothelial cancer, and be a predictor for cancer diagnosis. In addition, we are interested to know if the association could be further linked to specifically bladder cancer and/or UTUC. In the results, the GG genotype in *CCND1* G870A was associated with a decreased risk for urothelial cancer, compared with the AA type. In addition, this was specifically observed in bladder cancer, not in UTUC (Table I). Furthermore, the AG genotype was an interesting bi-directional predictor for muscle-invasiveness in urothelial cancers. The AG genotype in *CCND1* G870A was associated with a decreased risk for muscle-invasive bladder cancer, while in UTUC it was associated with an increased risk for muscle invasion (Table II). Since the patients of UTUC were not easily collected, and the limited sample size can not exclude the possibility of neither false positive nor false negative findings after the stratification, the potential role for the G allele in *CCND1* G870A as a diagnosis predictor may need to be clarified in the future with a larger population.

In 2002, Wang *et al* firstly indicated the possibility that the *CCND1* 870 AA genotype confers elevated risk for bladder cancer in native Japanese, with more pronounced risk among non-smoking cases and for bladder cancer of higher grade and stage (20). Based on reexamining their findings, Cortessis and his colleagues had brought up their negative findings in investigating a Caucasian population in California of USA in the next year (17). In 2004, Ito *et al.* (teammates of Wang) further examined the influence of *CCND1* G870A genotypes on prognostic parameters such as the recurrence of superficial cancer and survival with invasive cancer rate (18). They found that in patients with superficial bladder cancer, the occurrence of primary carcinoma in situ was significantly greater in patients with the AA genotype compared with those with the GA or GG genotypes (18). Almost at the same time, Sanyal and his colleagues conducted a case-control study investigating the roles of genotypes of several genes involved in DNA repair, metabolism and cell-cycle regulation in a moderate sized population Caucasian. In their study, no significant differences for genotype distributions and allele frequencies of *CCND1* G870A between the bladder cancer cases and the controls were observed (21). In 2010, Yuan and his colleagues had collected a moderate case-control sample population in Nanjing city in China, examining the contribution of *CCND1* G870A genotyping to the etiology of bladder cancer in China (22). In their study, a significantly increased risk of bladder cancer

was associated with 1.54-fold increased risk for those with GA/AA genotypes of *CCND1* G870A, compared with the GG genotype, particularly among subgroups of age ≥65 years, male, and smokers. Further, the G870A polymorphism was significantly associated with risk of developing superficial bladder cancer (grade 1) (22). All these above findings were investigating the genetic role of *CCND1* G870A in bladder cancer, but not in UTUC. In addition, none of them was investigating the Taiwanese population, which is very genetic homogenous and conserved, and suffering from serious bladder cancer and UTUC. The inconsistence among them may mainly be due to different ethnicities in various genetic backgrounds, environmental exposures, and diet cultures under the investigations. All the previous findings together with ours may turn to a temporary conclusion that the variant 870GA/AA genotypes were associated with an increased risk of bladder cancer in Asians (China, Japan and Taiwan), but not in Caucasians. All the literature investigating the genetic role of *CCND1* G870A were concisely summarized in Table III for brief comparison (Table III).

Bladder cancer and UTUC can be of two types, non-muscle invasive and muscle-invasive types, depending on pathological findings. Recurrence and progression are the most serious risks following treatment of the former, whereas local invasion and distant metastasis are life-threatening issues in patients with the

later. It is interesting that the genotype of *CCND1* G870A can not only be a prognosis

for both bladder cancer and UTUC in Taiwan but also can predict a different cancer

progression outcome for bladder cancer (AG for lower risk for muscle invasiveness),

and UTUC (GG for higher risk for muscle invasiveness) (Table II). The limited and

not easily collectible sample size provided us the biphasic but interesting pilot result,

and encouraged us to re-examine our results in a larger population for the

conformation.

It has been known that *CCND1* 870 AA genotype will influence the

alternatively spliced forms of the *CCND1* mRNA and produce variant transcript-b

(16). The transcript-b may have a longer half-life since it lacks the PEST

(praline-serine-threonine)-rich region for rapid degradation (33), hence may alter the

normal regulation of the cell cycle. Under such circumstances, the *CCND1* A allele

could exert an alterations of the behaviors of the cancer cells during different stages of

carcinogenesis. In this paper, we have found that the *CCND1* G870A was associated

with urothelial cancer, and specifically with bladder cancer. While in different

microenvironments, the overall effects of this subtle polymorphism may cause

different muscle-invasive susceptibilities to bladder cancer (protective) and UTUC

(risky).

In conclusion, this study suggested that *CCND1* G870A GG genotype was

positively associated with a lower risk of urothelial cancer, especially with UTUC. In addition, the AG heterozygous patients of bladder cancer were of lower risk and the GG homozygous patients were of higher risk for their muscle-invasiveness, respectively. These findings suggested that G allele could be a very interesting predictor in lower urothelial (especially bladder) cancer susceptibilities, and muscle-invasiveness, with a biphasic cancer progression in bladder cancer (protective) and UTUC (risky).

**Figure legend**

Fig. 1 (a) Restriction map of *CCND1* G870A genotypes. On digestion with *ScrF*I, the PCR

product arising from the G allele was cut into fragments of 145 and 22 bps, whereas A allele

was uncut 167 bp. (b) Electrophoregram of PCR-RFLP of *CCND1* G870A. Lane 1, 50 bp

MW marker; Lane 2, 167 bp PCR product; Lane 3, GA heterozygote; Lane 4, GG

heterozygote; Lane 5, AA homozygote.

Table I. Characteristics and *CCND1* G870A genotypes among bladder cancer, upper tract urothelial cancer cases and healthy controls.

| *CCND1* genotype | Control (n=243) | All Cases (n=171) | OR (95% CI) | *P*-value | Bladder (n=101) | OR (95% CI) | *P*-value | Upper (n=70) | OR (95% CI) | *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|
| AA | 78 | 72 | 1.000 (Ref) | | 42 | 1.000 (Ref) | | 30 | 1.000 (Ref) | |
| AG | 116 | 79 | 0.74 (0.48~1.13) | 0.189 | 50 | 0.80 (0.49~1.32) | 0.442 | 29 | 0.81 (0.42~1.56) | 0.176 |
| GG | 49 | 20 | **0.44 (0.24~0.81)\*** | **0.008\*** | 9 | **0.34 (0.15~0.76)\*** | **0.008\*** | 11 | 0.58 (0.27~1.27) | 0.194 |
| AG+GG | 165 | 99 | **0.65 (0.43~0.98)\*** | **0.039\*** | 59 | 0.66 (0.41~1.07) | 0.107 | 40 | 0.63 (0.37~1.09) | 0.116 |

OR: odds ratio, 95% CI: 95% confidence interval, Ref, reference.

*: statistical significant

Table II. Association between different *CCND1* G870A polymorphic genotypes and pathological characteristics in urothelial carcinoma.

| | Bladder cancer | | | Upper tract cancer | | |
|---|---|---|---|---|---|---|
| | AA | AG | GG | AA | AG | GG |
| **Stage** | | | | | | |
| Non-muscle invasive | 20 | 38 | 5 | 17 | 13 | 2 |
| Muscle invasive | 22 | 12 | 4 | 13 | 16 | 9 |
| OR | 1 (ref) | **0.29\*** | 0.73 | 1 (ref) | 1.61 | **5.88\*** |
| 95% CI | | **0.12-0.70\*** | 0.17-3.09 | | 0.58-4.50 | **1.08-32.01\*** |
| *P*-value | | **0.009\*** | 0.727 | | 0.439 | **0.039\*** |
| **Grade** | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Lower grade | 16 | 21 | 5 | 12 | 11 | 3 |
| Higher grade | 26 | 29 | 4 | 18 | 18 | 8 |
| OR | 1 (ref) | 0.85 | 0.49 | 1 (ref) | 1.09 | 1.78 |
| 95% CI | | 0.37-1.97 | 0.11-2.11 | | 0.38-3.10 | 0.39-8.09 |
| *P*-value | | 0.831 | 0.460 | | 1.000 | 0.716 |

OR: odds ratio, 95% CI: 95% confidence interval, Ref, reference.

*: statistical significant

Table III. A brief summary of the reports investigating the role of *CCND1* G870A polymorphic genotypes in urothelial carcinoma.

| Disease | Author, year | Study subjects | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | Ethnic Country | Cases | Controls | Statistical Significance | Brief description |
| Bladder cancer | Wang, 2002 | Japanese | 222 | 317 | S | AA genotype is more risky than GG genotype |
| | Cortessis, 2003 | Caucasian | 515 | 612 | NS | |
| | Ito, 2004 | Japanese | 173 | 0 | S | AA genotype is more risky than GG genotype in primary carcinoma occurrence, but not in survival after radical cystectomy |
| | Sanyal, 2004 | Caucasian | 327 | 246 | NS | |
| | Yuan, 2010 | China | 402 | 402 | S | AA/GA genotype is more risky than GG genotype, especially in those ≥65 years old, |

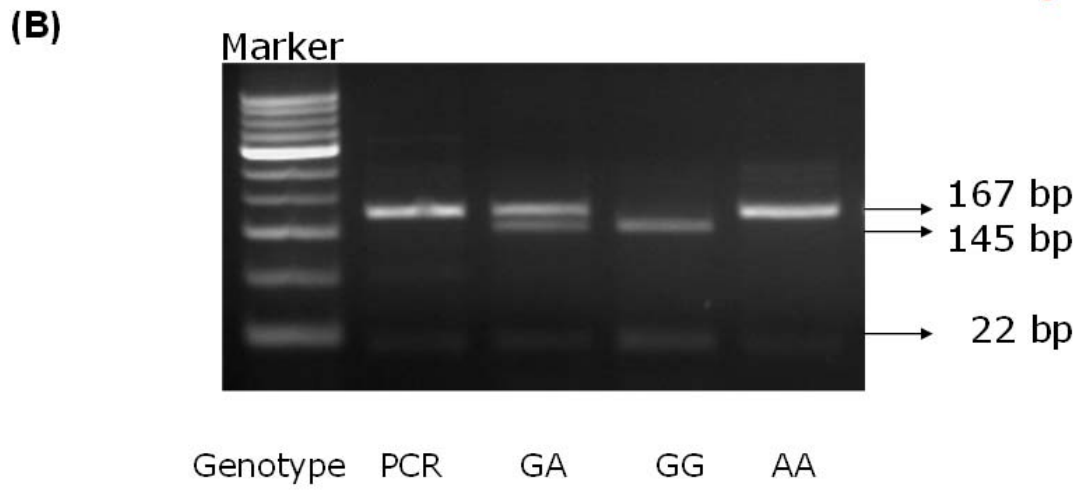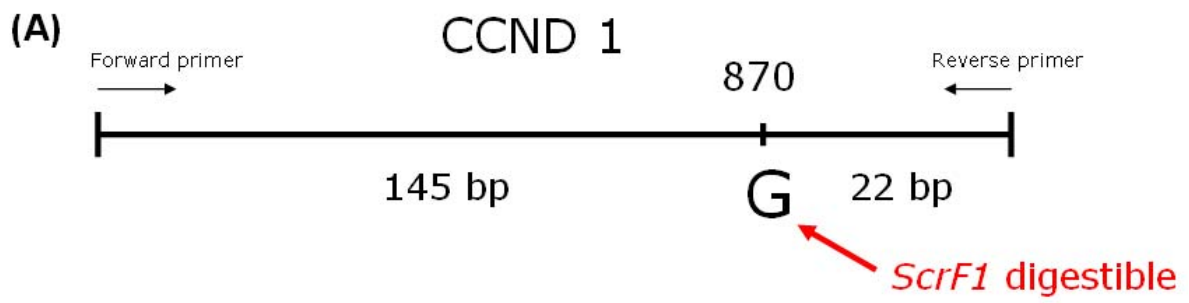| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | male, and smokers. |
| | Lin, 2011 | Taiwan | 101 | 243 | S | GG genotype is more protective than AA genotype in both cancer susceptibility and muscle invasiveness, but not in higher grade. |
| Utter tract urothelial cancer | Lin, 2011 | Taiwan | 70 | 243 | NS | |

S: statistically significant; NS: not statistically significant

**(A)**

CCND 1

Forward primer

870

Reverse primer

145 bp

G 22 bp

ScrF1 digestible

**(B)**



| Genotype | PCR | GA | GG | AA |

167 bp
145 bp
22 bp

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