Chinese Journal of Physiology 54(3): 196-202, 2011
DOI: 10.4077/CJP.2011.AMM123

Potential Role of *CCND1* G870A Genotype as a Predictor for Urothelial Carcinoma Susceptibility and Muscle-Invasiveness in Taiwan

Hui-Hui Lin[1, 2], Hung-Lung Ke[1, 3], Kuang-Hung Hsiao[1, 2], Chia-Wen Tsai[5], Wen-Jeng Wu[3, 4], Da-Tian Bau[5, 6, *], and Lin-Li Chang[1, 2, *]

[1]*Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung*
[2]*Department of Microbiology, Kaohsiung Medical University, Kaohsiung*
[3]*Department of Urology, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung*
[4]*Department of Urology, College of Medicine, Kaohsiung Medical University, Kaohsiung*
[5]*Terry Fox Cancer Research Laboratory, China Medical University Hospital, Taichung*
and
[6]*Graduate Institutes of Clinical Medical Science, China Medical University, Taichung*
Taiwan, Republic of China

Abstract

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 a common *CCND1* G870A genotype is associated with bladder cancer in Japan and China, but not in the Western World. There is neither a report about its role in Taiwan's population, nor its genetic role of *CCND1* G870A in another worldwide urothelial cancer, upper tract urothelial cancer (UTUC). Therefore, we aimed at investigating the role of *CCND1* G870A in both bladder cancer and UTUC in Taiwanese cohorts. The *CCND1* G870A genotypes of 171 (101 bladder cancer and 70 UTUC) patients and 243 control subjects were determined by PCR-RFLP and their correlation with clinical and histopathological data was evaluated. The genotype analysis results showed that *CCND1* GG genotype was associated with a lower risk overall in urothelial (*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 the AA genotype. In addition, patients carrying the AG genotype had a 0.29-fold lower odds ratio of muscle-invasive cancer procession (95% CI = 0.12-0.70) compared with those carrying the AA genotype in bladder cancer patients. Surprisingly, the GG genotype had a 5.88-fold higher odds ratio of muscle-invasive cancer procession (95% CI = 1.08-32.01) compared with those carrying the AA genotype in UTUC. No association between any *CCND1* G870A genotype and higher-grade risk was found. Our results suggest that the G allele of the *CCND1* G870A polymorphism may be a potential predictive and prognostic biomarker to distinguish between bladder cancer and UTUC in Taiwan.

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

Corresponding author: Lin-Li Chang, Department of Microbiology, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 807, Taiwan, ROC. E-mail: datian@mail.cmuh.org.tw; artbau1@yahoo.com.tw
*These two authors have equal contributions to this work.
Received: December 30, 2010; Revised: January 21, 2011; Accepted: February 18, 2011.
 ISSN : 0304-4920. http://www.cps.org.tw

Introduction

Cancers of the urinary system are among the most frequent malignancies worldwide. Amongst them, kidney and bladder cancers are more common. Renal cell carcinoma (RCC), accounting for the majority of kidney neoplasms, and transitional cell carcinoma (TCC) of the bladder and upper tract, are the fourth most frequent malignancy in males (15). In the West and Taiwan, the incidence ratios for TCC of the pelvis, ureter and bladder, are quite different. It is 3:1:51 in the West and 1:2.08:6.72 in Taiwan (8, 32). Upper tract urothelial cancer (UTUC) is relatively rare in the West and the unusually high incidences of UTUC in Taiwan make it valuable to study the high prevalence in Taiwan and the comparison of the counterpart findings in Western 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 (7, 19-23). A recent study has provided evidence that genetic polymorphisms may also predispose the development of cancer disease (24).

Cyclin D1 (*CCND1*) is a key regulator of G1-S cell cycle progression; overexpression of cyclin D1 is implicated in the etiology of several cancers including TCC of the bladder (27, 30, 33). In addition, *CCND1* was thought to play an important role in the early stage of urothelial tumorigenesis and has been shown to correlate with early recurrence, tumor differentiation and clinical outcome in bladder cancer (18, 28). The *CCND1* gene 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 (4). In recent years, several studies showed that the *CCND1* 870 AA genotype was associated with an increased risk and it has influenced the outcome for bladder cancer (9, 14, 16, 26, 31, 34). However, there is no literature investigating another type of urothelial cancer, UTUC.

Thus, this study aimed at exploring the association between the *CCND1* G870A genotype and the susceptibility of urothelial carcinoma, including both bladder cancer and UTUC, and the correlation of the *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) patients with TCC were recruited at Kaohsiung Medical University medical center from Jan 2006 to Dec 2007; all the patients were diagnosed with urothelial cancer by pathologic examination of specimens obtained by biopsy or surgical resection. The clinical and histopathologic information and cigarette smoking history were collected from patient charts and pathologic reports. The information was reviewed, and the data were entered into a database. The tumor stage was assigned according to the TNM staging system (12), and the pathologic grade was determined according to the World Health Organization criteria (10). Two hundred and forty-three healthy individuals, who had been matched with the patients by age and admitted to the same hospital for health checkup and had no previous diagnosis of urologic neoplastic disease or other malignancies, 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 the informed consent and this study was approved by the Human Research Committees of the participating hospitals. This study had also been reviewed by the Institutional Review Board (IRB) of Kaohsiung Medical University with the approval number KMU-IRB-950195.

Genotyping Conditions

Genomic DNA for analysis was extracted from blood specimens using proteinase K digestion followed by phenol-chloroform extraction as described previously (6). Genotyping for *CCND1* G870A of all subjects was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays (1-3). The 167-bp fragments containing the polymorphic nucleotide were amplified using the forward primer 5'-GTGAAGTTCATTTCCAATCCGC-3' and the reverse primer 5'-GGGACATCACCCTCACTTAC-3'. The following cycling conditions were performed: 5 min of initial denaturation at 95°C, 35 cycles of 30 sec of denaturation at 95°C, 30 sec of annealing at 54°C and 1 min of elongation at 72°C, and 7 min of final extension at 72°C. The PCR products were further digested with HaeIII (New England, Biolabs, Beverly, MA USA), and visualized by ethidium bromide-stained 3% agarose gel electrophoresis under UV light. On digestion with *ScrFI*, the PCR product arising from the A allele was uncut (167 bp), whereas the G allele was cut into fragments of 145 bp and 22 bp (Fig. 1). Sequences were confirmed by direct sequencing of 10% of the PCR samples, and the results were 100% concordant.

Statistical Analysis

To ensure that the controls used were representa-

Table 1. 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

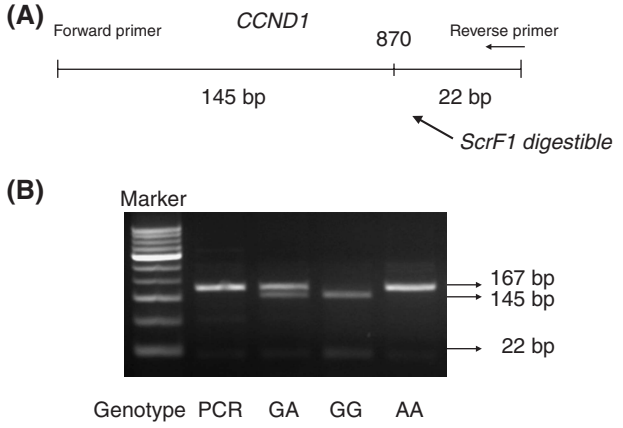

Fig. 1. (a) Restriction map of *CCND1* G870A genotypes. On digestion with *ScrF*I, the PCR product arising from the G allele was cut into fragments of 145 bp and 22 bp, whereas the A allele was the uncut length of 167 bp. (b) Electrophoregram of PCR-RFLP of *CCND1* G870A. Lane 1, 50-bp size marker; lane 2, the 167-bp PCR product; lane 3, a case of GA heterozygote; lane 4, GG heterozygote; lane 5, AA homozygote.

tive of the general population and to exclude the possibility of genotyping errors, 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 were 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 are presented in Table 1. The rationale and electrophoregram of PCR-RFLP of *CCND1* G870A are also presented in Fig. 1. Both allele distribution frequencies of the patient and the control groups fitted the Hardy Weinberg equilibrium. Compared with the *CCND1* G870A AA genotype, patients with the GG genotype tended to have 0.44-fold risk of urothelial cancer ($P = 0.008$; OR = 0.44, 95% CI = 0.24-0.81). Patients with the AG + GG genotype also had a significant 0.65-fold risk of urothelial cancer compared with individuals with the AA genotype ($P = 0.039$; OR = 0.65, 95% CI = 0.43-0.98). It seems that the G allele is a protective genetic factor for urothelial cancer. We further divided the patients into bladder cancer and UTUC subgroups and reevaluated their risks of urothelial cancer. Interestingly, bladder cancer patients with the GG genotype tended to have further lower risk of urothelial cancer ($P = 0.008$; OR = 0.34, 95% CI = 0.15-0.76) compared with those with the AA type. But this trend was not observed in the UTUC patients (Table 1).

The association of *CCND1* G870A genotypes with pathological characteristics in both bladder cancer and UTUC patients is presented in Table 2. 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 (13). However, the genetic factors are largely unknown. Our data showed that when compared with the AA genotype, subjects 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, subjects 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 in comparion with the AA genotype (Table 2). Second, patients

Table 2. 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.

with similar stage but different grades respond to treatment differently (17). Thus, all the patients of the early stage were genotyped and further stratified by their pathological stages and the risk was analyzed. The results showed that in both bladder cancer and UTUC, neither of the genotypes of *CCND1* G870A was positively associated with muscle-invasive risk (Table 2).

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 (29). Although intragenic somatic mutation of cyclin D1 in human diseases is rare, translocation, amplification and/or overexpression of the cyclin D1 gene are frequent events in selected tumor types. In the literature, the polymorphism in the cyclin D1 locus that may affect splicing has been implicated in increased cancer risk and poor outcome has also been reported (16). 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 poor progression in urothelial cancer (5, 25). In this study, we hypothesized that the *CCND1* G870A polymorphism may be associated with the risk of urothelial cancer, and may be a predictor for cancer diagnosis. In addition, we would be interested to know if the association could be further linked specifically to 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 1). 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 2). According to the information on the NCBI SNP website, the ratios of the *CCND1* G870A allele frequency in populations of Taiwan and China are very similar (G/A = 44.0%/56.0% in our Taiwanese controls and G/A = 45.6/54.4% in China). The A allele seemed to be the major allele in Taiwan and China populations. However, the ratio of allele of the *CCND1* G870A frequency in the Japan population is G/A = 61.1%/38.9%, quite different from the previously described Asian populations. Interestingly, in Caucasian population, the G/A ratio is 48.3%/51.7%, similar to Taiwan and China populations. Therefore, validation of our findings in other populations is warranted to understand the similarity and difference among various ethnic groups. Since the patients of UTUC were not easily collected, and the limited sample size could not exclude the possibility of false positive or 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 confered elevated risk for bladder cancer in native Japanese people with more pronounced risk among non-smoking

Table 3. Summary of reports investigating the role of *CCND1* G870A polymorphic genotypes in urothelial carcinomas

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.
	Gangwar, 2010	India	212	250	S	AA genotype is more risky than GG genotype, and had a joint effect with *MDM2* genotype on cancer risk.
	Present study	Taiwan	101	243	S	GG genotype is more protective than AA genotype in both cancer susceptibility and muscle invasiveness, but not in higher grade.
Upper tract urothelial cancer	Present study	Taiwan	70	243	NS	

S: statistically significant; NS: not statistically significant.

cases and for bladder cancer of higher grades and stages (31). Based on reexamination of their findings, Cortessis and his colleagues had shown negative findings in investigating a Caucasian population in California of USA in the following year (9). 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 (14). 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 (14). 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 moderately sized population of Caucasians. 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 (26). In 2010, Yuan and his colleagues had collected a moderate size case-control sample population in Nanjing city in China and had examined the contribution of *CCND1* G870A genotyping to the etiology of bladder cancer in China (34). 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 smokers. Furthermore, the G870A polymorphism was significantly associated with the risk of developing superficial grade 1 bladder cancer (34). In 2010, Gangwar and his colleagues conducted a similar study in North India and found that the AA genotype of *CCND1* G870A was associated with higher risk in intermediate bladder cancer stage and in smokers. The combined genotype of *MDM2* T309G and *CCND1* G870A can be a predictor for bladder cancer risk (11). All the above findings investigated the genetic role of *CCND1* G870A in bladder cancer, but not in UTUC. In addition, none of them investigatied the Taiwanese

population, which is genetically highly homogenous and conserved, and Taiwanese patients 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 investigation. All the previous findings together with ours may be taken as a provisional conclusion that the variant 870GA/AA genotypes are 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 summarized in Table 3 for comparison.

Bladder cancer and UTUC can be of two types, the non-muscle invasive and the 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 in patients with the latter. It is interesting that the genotype of *CCND1* G870A can be a prognosis for both bladder cancer and UTUC in Taiwan but can also predict a different cancer progression outcome for bladder cancer (AG for a lower risk for muscle invasiveness), and UTUC (GG for a higher risk for muscle invasiveness) (Table 2). The limited and not easily collected sample size provided us with the biphasic but interesting results in this pilot study; our results should be re-examined in a larger population for confirmation.

It has been known that the *CCND1* 870 AA genotype influences the alternatively spliced forms of the *CCND1* mRNA and produces variant transcript-b (4). The transcript-b may have a longer half-life since it lacks the PEST (praline-serine-threonine)-rich region for rapid degradation (25) and, hence, may alter the normal regulation of the cell cycle. Under such circumstances, the *CCND1* A allele could exert alterations of the behavior of the cancer cells during different stages of carcinogenesis. In this paper, we found that the *CCND1* G870A was associated with urothelial cancer, and specifically with bladder cancer. 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, the results of this study suggested that *CCND1* G870A GG genotype was positively associated with a lower risk of urothelial cancer, especially with bladder cancer. In addition, AG heterozygous patients of bladder cancer were of a lower risk and the GG homozygous patients of UTUC were of a higher risk for muscle-invasiveness, respectively. These findings suggested that G allele could be an interesting predictor in lower urothelial, especially bladder, cancer susceptibilities and muscle-invasiveness with a biphasic cancer progression in bladder cancer (protective) and UTUC (risky).

Acknowledgment

The work was supported by a grant from the Kaohsiung Medical University Hospital (KMUH 5D-43).

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