The Influence of Cyclin D1 A870G Polymorphism on Colorectal Cancer Risk and Prognosis in a Turkish Population

İLHAN YAYLIM-ERALTAN[1], SOYKAN ARIKAN[2], YEMLİHA YİLDİZ[1], CANAN CACİNA[1], H. ARZU ERGEN[1], GULAY TUNA[1], UZAY GÖRMÜŞ[1], ÜMİT ZEYBEK[1] and TURGAY İSBİR[3]

[1]*Department of Molecular Medicine, Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey;*
[2]*Surgery Clinic, Istanbul Research and Teaching and Research Hospital, Istanbul, Turkey;*
[3]*Department of Medical Biology, Medical Faculty Yeditepe University, Istanbul, Turkey*

Abstract. *Background: Cyclin D1, encoded by the gene CCND1, is a regulatory protein in the cell cycle transition from G_1 phase to S phase. A common polymorphism (A870G) at codon 242 affects splicing of the CCND1 transcript and may cause uncontrollable cellular growth. The present study was performed to test the association between A870G polymorphisms in the CCND1 gene and colorectal cancer risk and progression. Patients and Methods: The 870 A>G polymorphism in the cyclin D1 gene was genotyped in a Turkish colorectal cancer case-control population including fifty-seven cases (35 male, 22 female; mean age±SD: 59.33±13.7 years) and 117 controls (63 male, 54 female; mean age±SD: 54.4±12.2 years) using polymerase chain reaction- restriction fragment length polymorphism analysis. Results: Genotype frequencies of our patients and controls both confirmed to the Hardy-Weinberg equilibrium. There was no difference in the distribution of CCND1 genotypes and frequencies of the alleles A (59.6% versus 49.6%) and G (40.4% versus 50.4%) in the colorectal cancer patients and controls, respectively. Women homozygous for the cyclin D1 870 GG genotype showed an increased risk for developing colorectal cancer compared to those with the AG+AA genotypes and this result was statistically significant (OR 5.568, 95% CI 1.270-24.417, p=0.02). On the other hand, the cyclin D1 GA genotype was associated with distant metastasis (p=0.016). Conclusion: Our findings suggest that genetic variants of A870G might be associated with distant metastasis and also gender.*

Correspondence to: Associate Professor Ilhan Yaylım-Eraltan, İstanbul Üniversitesi DETAE 34390 P.O. Box 7 Çapa/İstanbul, Turkey. Tel/Fax: +90 2126351959, e-mail: yaylimyaylim@istanbul.edu.tr

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Cyclin D1 (*CCND1*) is a key cell cycle regulatory protein at the G_1/S checkpoint of the cell cycle. It has been reported that amplification of CCND1 and altered expression of the protein are associated with cell proliferation and poor prognosis in a variety of tumors, including head and neck, colon, breast, lung (1-5). Increased expression of cyclin D1 has been observed in 40% of colorectal cancer cases, 30% of human adenocarcinomas and adenomatous polyps of the colon (6-8). *CCND1* mRNA is alternatively spliced to produce two transcripts (a and b). It is known that the main difference in the cyclin D1 protein encoded by the two transcripts is in the C-terminal PEST-rich region (destruction box) encoded by exon 5 (9, 10). Betticher and colleagues identified a single nucleotide polymorphism (A870G) in the *CCND1* gene (9). This polymorphism at codon 242 , the boundary of exon 4 and intron 4, affects alternative splicing such that exon 5 is not expressed in the A allele. Since exon 5 is involved in rapid turnover, the variant cyclin D1 corresponding to the A allele may have a longer half-life than the G allele. It has been suggested this might allow the damaged cells to pass through the cell cycle and then progress to cancer (11, 12). However, each of these allelic variants may not always have the same effects on cancer progression. While homozygosity for the G allele was related to improved prognosis in patients with non-small lung cancer (NSCLC), the same genotype corresponded with reduced disease-free interval in both laryngeal and pharyngeal carcinomas (9, 13). In this case control study, our aim was to investigate the influence of *CCND1* genotype on the genetic susceptibility and progression of colorectal cancer in a Turkish population.

Materials and Methods

Patient selection and clinical investigation. Fifty-seven patients with colorectal adenocarcinomas and 117 healthy controls were included in the study. All patients, diagnosed by colonoscopy or sigmoidoscopy and histologically confirmed, were treated at the Istanbul Education and Research Hospital, Surgery Clinics between

2006 and 2008. The mean (±SD) age of colorectal cancer patients was 59.33±13.71 years. The diagnoses of the patients were determined by endoscopic radiologic and operative findings and confirmed by pathological examination. The blood samples were collected from the patients before any treatment had been started (chemotherapy or radiotherapy). The control group was selected from patients attending the general surgery and orthopedic clinics of the same hospital and who were treated for non-neoplastic diseases such as inguinal hernia or trauma. The mean (±SD) age of the control group was 54.4±12.2 years. All the participants, after giving written informed consent, completed a structured self-administered questionnaire in order for us to collect demographic data. A standardized questionnaire was administered to collect data concerning age, sex, family history of colorectal cancer, and family history of any kind of cancer for 57 colorectal cancer patients from whom we obtained blood samples. The study protocol was approved by the local medical ethical committee.

Pathological staging information on all colorectal cancer diagnoses were confirmed by manual review of the pathology reports and clinical charts. Each tumor stage was coded as described by the AJCC sixth edition according to the TNM stage (14). The cancer-specific data for each patient contained the tumor location (right colon, transverse colon, left colon, rectum), tumor grade (well-differentiated, moderately differentiated, poorly differentiated, anaplastic or undifferentiated), specific histology (adenocarcinoma, mucinous, signet ring cell), and metastases detected by (PET-CT) scan at presentation (15, 16).

DNA extraction and genotyping. Genomic DNA samples of the participants were extracted from peripheral blood leukocytes using the method of Miller *et al*. based on sodium dodecyl sulphate lysis, ammonium acetate extraction, and ethanol precipitation (17). Primers (forward :5'GTG AAG TTC ATT TCC AAT CCG C3') and (reverse :5'GGG ACA TCA CCC TCA CTT AC3') were used to amplify a 167 bp DNA fragment from the boundary of exon 4 and intron 4 including G/A polymorphism in codon 242. Each PCR reaction (25 μl) final volume consisted of 1 μl of sample DNA, 1.5 mM $MgCl_2$, 0.4 μM of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 0.16 mM of deoxnucleotide triphosphate (MBI Fermentas), and 1 unit of Taq polymerase (MBI Fermentas). The reaction mixture was initially denatured at 94˚C for 5 minutes, followed by 35 cycles with denaturation steps at 94˚C for 45 seconds, annealing at 55˚C for 45 seconds, and extension at 72˚C for 45 seconds. The PCR programme was completed by a final extension cycle at 72˚C for 5 minutes. Ten microliters of each PCR product (167 bp) were digested with 15U *NciI* (MBI Fermentas) at 37˚C for 3 hours and visualized by electrophoresis on 2.5% agarose containing 0.5 mg/ml ethidium bromide (18). If the G allele is present, the PCR product is cut to produce fragments of 22 and 145 bp by *NciI*, whereas the product from the A allele is not cut by this restriction enzyme.

CCND1 A870G polymorphism was typed by visualization under ultraviolet light and photographing with a Polaroid camera. The *CCND1* A870G alleles were identified in each sample (18). For quality control, 55 randomly selected DNA samples (31.6% of all samples) were subject to repeat PCR and genotyping and no discrepancies were detected.

Statistical analysis. All statistical analyses were carried out using SPSS version 7.5 for Windows (SPSS Inc, Chicago, USA). Numeric values were analysed by Student's *t*-test. *CCND1* genotype frequencies were correlated between patient and control groups using the chi-squared test, as was clinicopathological data for the colorectal cancer group. The Hardy-Weinberg equilibrium test was used to detect selection bias or experimental error in the genotyping. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measurement of the association between *CCND1* genotypes and colorectal cancer risk. The threshold for significance was $p<0.05$.

Table I. *Characteristics of the cancer patient and control groups*.

Parameter	Colorectal cancer patients	Control group
Gender		
Male	35 (61.4)	54 (46.2)
Female	22 (38.6)	63 (53.8)
Age (years)		
Mean±SD	59.33±13.7	54.4±12.2
Range	23-85	20-87
Median	61	51
Ever-smokers	6 (10.5)	9 (9.8)
Consuming alcohol	3 (5.4)	3 (3.3)
Family history of cancer	5 (5.3)	0 (0)

SD, Standard deviation; Data are numbers with percentages in parentheses unless otherwise indicated.

Results

In this study, we determined the *CCND1* A870G polymorphism in both colorectal cancer patients and controls by using PCR-RFLP analysis. The demographic characteristics of the study groups are presented in Table I. There were no significant differences between these characteristics in the study groups. The *CCND1* G870A genotypes were in Hardy-Weinberg equilibrium, as demonstrated by the lack of any significant difference between their observed and expected frequencies, in both the colorectal cancer (χ^2=0.024; p=0.88) and control group (χ^2=0.08; p=0.781). The frequencies of A and G alleles were 59.7% and 40.3% and 49.6% and 50.4%, in the patients and healthy controls, respectively. With the use of the χ^2 test, the difference of the distribution of the three *CCND1* A870G genotype and allelic frequencies between colorectal cancer patients and controls were not statistically significant (p=0.203 and p=0.771, respectively). Our results do indicate that individuals carrying the AA genotype have an increased risk for development of colorectal cancer (OR 1.466, 95% CI 0.909-2.366) as compared with individuals carrying the GG genotype (Table II). We also found that the homozygous G allele was more frequent in women with colorectal cancer than in those without (OR 5.568, 95% CI 1.270-24.417) and this value was statistically significant

Table II. *Association of CCND1 A870G polymorphism with colorectal cancer risk.*

Genotype	Patients n (%)	Controls, n (%)	OR (95% CI)	*p*-Value
GG	9 (15.8)	29 (24.8)	1.00 (reference)	-
AA	20 (35.1)	28 (23.9)	1.404 (0.980-2.012)	0.080
AG	28(49.1)	60 (51.3)	0.856 (0.648-1.130)	0.251
AA+AG	48 (84.2)	88 (75.2)	1.120 (0.961-1.305)	0.178†
AG+GG	37 (64.9)	89 (76.1)	0.853 (0.687-1.059)	0.122‡

p-Values obtained by chi-square test. †Wild-type genotype (GG) compared to combination of heterozygous and homozygous variant genotypes (AG+AA); ‡homozygous variant genotype (AA) compared to combination of wild-type and heterozygous genotypes (GG+GA).

Table III. *Distribution of CCND1 G870A genotype with clinicopathological features in colorectal cancer patients.*

	CCND1 genotype			*p*-Value
	AA N (%)	AG N (%)	GG N (%)	
Gender				
Male	16 (45.7)	17 (48.6)	2(5.7)	0.013
Female	4(18.2)	11 (50)	7 (31.8)	
T stage				
T3+T4	15 (39.5)	16 (42.1)	7 (18.4)	0.322
T1+T2	5 (26.3)	12 (63.2)	2 (10.5)	
Lymph node status				
N(+)	10 (33.3)	16 (53.3)	4 (13.3)	0.769
N(–)	10 (37)	12 (44.4)	5 (18.5)	
Distant metastasis (+)				
Yes	2 (18.2)	9 (81.8)	0 (0)	0.044
No	18 (39.1)	19 (41.3)	9 (19.6)	
Perineural invasion				
+	8 (47.1)	9 (52.9)	0 (0)	0.087
–	12 (30)	19 (47.5)	9 (22.5)	
Angiolymphatic invasion				
+	8 (33.3)	13 (54.2)	3 (12.5)	0.765
–	12 (36.4)	15 (45.5)	6 (18.2)	

p-Values obtained by chi-square test.

(*p*=0.02). There was no significant difference between the distribution of *CCND1* A870G genotypes and tumor stage and lymph node status in our colorectal cancer patients (*p*=0.322 and *p*=0.769, respectively). The presence of distant metastasis in patients with heterozygous AG was more frequent as compared with patients carrying the AA and GG genotypes and this difference was statistically significant (OR 1.4981, 95% CI 1.272-3.085; *p*=0.016) (Table III). Another relationship was also observed between patients with perineural invasion: in these patients, the frequency of the homozygous G allele was higher than those without (OR 1.290, 95% CI 1.092-1.525; *p*=0.046). Colorectal cancer patients carrying the *CCND1* A allele had a 1.29-fold increased risk for perineural invasion (*p*=0.046). Angiolymphatic invasion was present in 24 (42.1%) cases and 33 (57.9%) cases respectively. These parameters were not found to have any statistically significant meaning for different genotypes. Furthermore, the *CCND1* genotypes were not associated with age, smoking status, family history of any kind of cancer or tumor location.

Discussion

Colorectal cancer has become a highly prevalent malignancy in recent decades. Effective management of colorectal cancer depends on early detection. Progress in this aspect would be by improving diagnostic and prognostic tools and also in encouraging patients to present early (19). Genetic factors are thought to play an important role in the development of colorectal cancer. There are many studies focused on the relationship between genetic polymorphisms and risks of colorectal cancer. However, the overall results from more than 2000 studies are inconsistent. It is known that tumorigenesis of colorectal cancer is multistepped, and cyclin D1 protein may be involved in one of these multiple pathways (20, 21). *CCND1* is an important cell cycle regulatory protein, the overexpression of which is often found in human tumors and is associated with cell proliferation and poor prognosis (8). A common adenine-to-guanine substitution polymorphism (A870G) at codon 242 in exon 4 of the *CCND1* gene has impacts on risk of the early-age onset in several malignant neoplasms, including colorectal cancer (20). It is known that this polymorphism affects splicing such that exon 5 is not expressed in the A allele. Since exon 5 is involved in rapid turnover, the variant cyclin D1 corresponding to the A allele may have a longer half-life (12, 22). Many case-control studies reported that patients with *CCND1* A allele or AA genotype have an increased risk and poor disease outcome in a number of cancer types, such as nasopharyngeal carcinoma (23) and cervical cancer (24). Our results are similar to earlier studies which reported that the A allele and AA genotype have a significant association with cellular proliferation. However, there are some reports with contrary results. Some studies have also implicated the G allele as a risk allele, or did not find a risk association at all (25-27). Despite the functional relevance of the *CCND1* A870G single nucleotide polymorphism, published results on its association with colorectal cancer are inconsistent (8, 28, 29). Recently, Pabalan *et al*. (30) conducted a meta-analysis on the association between A870G polymorphism and the risk of

cancer. They combined sixty studies representing data for 18,411 cases and 22,209 controls. They reported that individuals homozygous for A allele (AA) were found to be associated with significantly increased cancer risk (in overall sample, OR 1.23; Caucasians, OR 1.16; and Asians, OR 1.26). They suggested that the independent small risk associated with the *CCND1* A870G polymorphism is not clinically useful. However, its interaction with other genetic variants and environmental factors has been shown to be associated with further increase in cancer risk (OR 1.6-7.1). Most interestingly, Bala and Peltomaki (31) were unable to confirm the observation reported by Kong *et al*. (32), in which the dominant allele A was related to an increased age-associated colon cancer risk. They reported that the age at onset of colon cancer was lower by 5-6 years in both types of homozygotes (AA and GG) as compared with heterozygotes (AG). They suggested that the relative abundance of a and b transcripts may modify the age at onset of colon cancer. The effect of *CCND1* A870G polymorphism on the risk of colorectal cancer is likely to vary in different racial and ethnic groups with different allelic frequency of the A allele. In this first study of associations between the *CCND1* A870G polymorphism and colorectal cancer in a Turkish population, the frequency of 49.6% for the A allele in our population was similar to the previous reports obtained for Caucasians (30-34). In the present study, we did not observe any association between the risk of colorectal cancer and *CCND1* A870G polymorphism. This is in agreement with some previous reports on head and neck cancer (13) and colorectal cancer (27). Although we were unable to find a significant result, a trend towards the AA genotype being related to an increased risk of colorectal cancer compared to the GG genotype was observed. In an analysis of our cancer group, the influence of age, smoking, family history of colorectal cancer did not differ significantly according to *CCND1* genotype. Zheng *et al*. reported that the AA genotype was associated with an increased risk of colorectal cancer at a younger age and sex. They reported that AA variant genotype was associated with a >3-fold increased risk in individuals who were ≤50 years old and females (35). Kong *et al*. (32) also showed that patients with the AA or AG genotype were 2.46 times more likely to develop colorectal cancer at any age than were patients with GG genotype. On the other hand, Huang *et al*. reported that the effect of AA/AG genotype on colorectal cancer risk was statistically significant for male patients. We could not find any correlation between age and *CCND1* G870A genotypes. However, there was an association between female gender and GG genotype. In our female patients, we observed that the frequency of the GG genotype was higher than patients with AG+AA genotypes. Schernhammer *et al*. performed a hospital-based prospective study of large size. They suggested that the GA or AA genotype may augment the various influences of estrogen in decreasing the risk of colorectal cancer (36). It has been suggested that physiological levels of estrogen may affect cyclin D1 expression and can stimulate cell proliferation (20, 37).

These conflicting results obtained from experimental findings may be due to the tissue-specific transcription regulation of *CCND1*. This may influence the effect of cyclin D1 genotype on tumor behaviour in different cell types. When compared with clinicopathological data, *CCND1* genotype was unrelated to the stage of disease, tumor size, lymph node metastasis or the site of tumor within the colorectum. These observations are similar to the results reported by McKay *et al*. (38). They detected cyclin D1 overexpression in 26% colorectal tumors and 38% of secondary lymph node tumors. They also found cyclin D1 to be overexpressed in lymph node tumors at a higher frequency than in the primary lesions, which may suggest a role for this protein in the metastatic process. Forones *et al*. (39) reported that the GG genotype was associated with an increased risk of metastatic colorectal cancer. In our study, stratification of cases according to clinical stages showed that the AG genotype was significantly frequently observed in association with a distant metastatic status in comparison with AA + GG genotypes. An interesting finding in our study was the elevated risk for perineural invasion in colorectal cancer patients who carried the GG genotype. Colorectal cancer patients carrying *CCND1* GG genotype had a 1.29-fold increased risk compared with those with the AA and AG genotypes. Perineural invasion has been demonstrated to be one of the important prognostic factors in patients with colorectal cancer (40).

The limitations of our study are as follows. Firstly, this study is a case-control study at a single hospital. Although a selection bias may have occurred, the *CCND1* A870G genotype frequencies of controls and patients in this study were in Hardy-Weinberg equilibrium. Secondly, we did not determine the level of CCND1b according to *CCND1* genotypes. Although, the sample size of our study was small, we reported here that the increased risk of distant metastasis and perineural invasion were associated with *CCND1* A870G polymorphism. In addition, based on our results, CCND1 polymorphism may modulate suspectibility to colorectal cancer differently in different genders. Further larger-scale studies are clearly warranted to confirm these associations between CCND1 A870G polymorphism and risk or progression of colorectal cancer.

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