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NOTE

Relationship between cyclin D1 (A870G) gene polymorphism and lung cancer

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The roles of many genes in the pathophysiology of lung cancer have been investigated in different studies. Cyclin D1 (CCND1) gene plays a significant role in the transition from G1 to S phase of the cell cycle and in the phosphorylation of retinoblastoma tumor suppressor protein. In this study, we aimed to identify the relationship between CCND1 A870G gene polymorphism with lung cancer. CCND1 A870G genotypes were determined in 75 patients with lung cancer and in 65 control subjects. DNA was isolated from blood samples and then CCND1 A870G gene polymorphism was identified using PCR and RFLP assay. The distribution of CCND1 A870G polymorphism did not show any significant differences in all lung cancer patients and controls. There was no correlation between CCND1 A870G polymorphism and histopathological findings. However, the AA + AG genotype was significantly higher in metastatic patients, when compared with non-metastatic patients. Thus, the results show that CCND1 gene polymorphism may be a predictor for detecting patients with poor survival who having metastatic disease.

Keywords: Lung cancer, Cyclin D1, Gene polymorphism.

Lung cancer is a common form of all human cancers in the world. It is the most common cause of cancer deaths in men and is the second most common cause of cancer deaths after breast cancer in women[1]. Smoking is responsible in 80-90% of patients with lung cancer, while in 10-20% cases, lung cancer is associated with other factors, such as age, sex, radiation, lung disease, nutrition and genetic[2].

Cyclin D1 (CCND1) gene, located on chromosome 11q13 encodes CCND1 protein, which consists of 295 amino acids and plays an important role as a regulator of cell cycle[3-5]. Retinoblastoma protein (pRb) has been identified as a tumor suppressor protein, which is the product of retinoblastoma gene[6]. Cell cycle progression through first gap phase (G1) to synthesis phase (S) requires inhibition of pRb activity by phosphorylation with CDK4 and cyclin D1 (CCND1). The CCND1/CDK4 complex is phosphorylated by CDK-activating kinase (CAK). pRb remains phosphorylated throughout S, G2 and M phases. Phosphorylation of pRb leads to separation of E2F (E2 promoter-binding protein dimerization partners) from the pRb/E2F complex. Dissociated E2F induces transcription of cyclin E and other genes which are required for entry into S phase[7,8].

Mutations, amplification and overexpression of CCND1 gene, which alters cell cycle progression, are observed frequently in a variety of tumors and may contribute to tumorigenesis[9-13]. A single base polymorphism (G870A) in exon 4 of CCND1 gene increases alternative splicing[14]. Normally, presence of the G870 allele leads to splicing into transcript a, which consists of exons 1-5 and encodes CCND1. Exon 5 comprises Thr 286, a site which is required for CCND1 nuclear export by Crm1. As an alternative transcript a, the presence of A870-allele favors splicing into transcript b that encodes for cyclin D1b. Cyclin D1b lacks exon 5-encoded Thr 286 and has been shown to accumulate in the nucleus and induce malignant transformation in normal cells[15-17]. In this study, we have investigated the relationship between CCND1 (A870G) gene polymorphism and lung cancer.

Materials and Methods

Peripheral venous blood samples were collected from newly diagnosed 75 patients with lung cancer, who were not treated with chemotherapy or radiotherapy before. Control samples were obtained from 58 healthy individuals having similar age. Approval for the study was obtained from Ethic Committee of Trakya University Faculty of Medicine.

Peripheral venous blood samples were collected into EDTA-containing tubes and stored at -20^{0}C until being analyzed. DNA was isolated from blood samples by Vivantis blood DNA kits (Vivantis, Subang Jaya, Selangor Darul Ehsan, Malaysia). DNA

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purity and quantity were assessed by their absorbance values by spectrophotometer (Shimadzu UV-1208) and checked by 0.8% agarose gel electrophoresis.

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods were used to determine genotype CCND1 A870G gene polymorphism, as described in an earlier study[18]. PCR primers for the CCND1 codon 242 (F: 5'-AGTTCATTTCCAATCCGCCC-3' and R: (5'-TTTCCGTGGCACTAGGTGTC-3') were used to generate a 212 bp product containing polymorphic site. The primer designs were based on the genomic DNA sequences (GenBank accession no. AF511593).

The CCND1 gene was amplified by PCR in a 25 µl PCR reaction mixture containing 0.2 µg of genomic DNA, 0.5 µmol/L of each primer, 0.2 mmol/L of each dNTP, 2.5 mmol/L MgCl2, 1X Taq buffer without $MgCl_2$ (500 mM KCl, 100 mM Tris-HCl, 0.1% Trion X-100) and 1.5 Units of Taq DNA polymerase (Vivantis, Subang Jaya, Selangor Darul Ehsan, Malaysia). Amplification was performed with a Techne DNA Thermal Cycler (TechGene) with initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C, annealing at 60°C, extension at 72°C for 1 min each and a final extension at 72°C for 7 min. The PCR product was digested with 5 units of MspI restriction enzyme (Fermantas Life Sciences) at 37°C overnight and run on 2.5% agarose gel. The wild genotype (GG) digested with MspI yielded three fragments of 141, 37 and 34 bp. The mutant genotype (AA) two fragments of 175 bp and 37 bp were produced. However, in heterozygous genotype (GA), all four bands were present. This assay illustrated in Fig. 1 when depicts the actual bands obtained after electrophoresis of digested product.

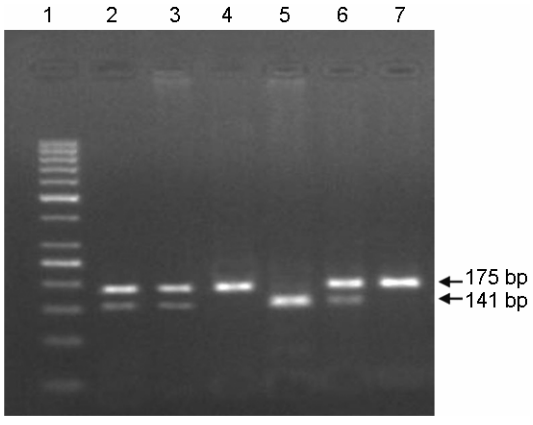

Fig. 1—RFLP analysis of CCND1 A870G gene polymorphism [Lane 1, 50 bp DNA marker; lanes 2, 3 and 6, AG heterozygous (175, 141, 37 and 34 bp); lane 5, GG homozygous (141, 37 and 34 bp); and lanes 4 and 7, AA homozygous (175 and 37 bp). The 37 bp and 34 bp bands are not visible on the 2.5% EtBr stained agarose gel]

Statistical analysis was performed using SPSS, version 15.0. (SPSS, Chicago, IL). Normality of the distribution was checked by using the Kolmogorov-Smirnov test. The observed genotype frequencies were used to test Hardy-Weinberg equilibrium (HWE) and the $\chi 2$ test. Statistical difference was considered significant for p values less than 0.05.

Results and Discussion

The demographic variables, such as age, sex and histological subtypes and genotypes for patients and controls are summarized in Table 1. The median age was 61 yrs in the patient group and 59 yrs in the controls. All patients had smoking history. The pathology was non-small cell lung carcinoma (NSCLC) in 61 (81.3%) patients. The frequencies of AA, AG and GG genotypes were 33.3, 49.4 and 17.3% in the patients group and 25.9, 53.4 and 20.7%

Table 1—Patients and controls characteristics

	Patients (n = 75) (%)	Controls (n = 58) (%)	p
Age (Median)	61	59	NS
Sex			
Male	72 (96)	55 (94.8)	NS
Female	3 (4)	3 (5.2)	
Histological type			
Non-small cell lung cancer (NSCLC)	61 (81.3)		
Squamous cell carcinoma (SCC)	22 (29.3)		
Adenocarcinoma	8 (10.7)		
Unclassified NSCLC	31 (41.3)		
Small cell lung cancer	14 (18.7)		
Metastasis status			
Non-metastatic (Stage I-III)	39 (52.0)		
Metastatic (Stage IV)	36 (48.0)		
Genotypes			
AA	25 (33.3)	15 (25.9)	NS
AG	37 (49.4)	31 (53.4)	
GG	13 (17.3)	12 (20.7)	
Allele			
A	87 (58.0)	61 (43.6)	0.02
G	63 (42.0)	79 (56.4)	

NS; no significance

in the control group, respectively. There was no difference in the frequencies of genotypes between patient and control groups. Although AA genotype was more frequent in the patient group (33.3% *versus* 25.9%), the difference was not significant. There was no correlation between AA genotype and lung cancer. But, the A allele frequency for A870G polymorphism was greater in patient group (p = 0.02).

Distant metastases were observed in 36 (48.0%) patients and most metastatic site was brain in 18 (50%) patients. Other metastasis sites were bone, adrenal gland and liver. Using the GG genotype as a reference, distribution of the A870G genotypes was not different in the non-metastatic patients from that of metastatic patients. But, the A allele (AA + AG *vs* GG, dominant model) was statistically higher in the metastatic patients in our study (p = 0.048) (Table 2).

CCND1 plays an important role in the transition from G1 to S phase[2-4]. Some studies have investigated the association between the CCND1 A870G polymorphism and the risk of lung cancer[15,18,19]. Qiuling *et al*.[19] reported the frequency of heterozygote AG and homozygote mutant AA allele 46.7% and 22%, respectively in patient group and 53% and 25.9%, respectively in control group; however, they could not show any significant differences between patient and control groups. Similar results were reported in another study and no significant difference was found between groups[18]. In our study, the frequency of heterozygote AG and homozygote mutant AA allele was 49.4% and 17.3%, respectively in patient group and 53.4% and 20.7%, respectively in the control group and there was no significant difference between patient and control groups.

In our study, no correlation was found between CCND1 gene polymorphism and histological subtypes, in agreement with the two above-mentioned studies. Although relationship has been shown between CCND1 gene polymorphism and lung cancer in patients who have smoking history in these studies[18,19]. we could not evaluate its' relation with smoking status because all of our patients had the smoking history.

Table 2—Stratification analysis of cyclin D1 gene polymorphism by metastasis status

		Metastasis		
		Yes (n, %)	No (n, %)	p
Genotype	GG (ref)	3 (8.3)	10 (25.6)	
	AG	19 (52.8)	18 (46.2)	NS
	AA	14 (38.9)	11 (28.2)	NS
	AA + AG	33 (91.7)	29 (74.4)	0.048

NS; no significance

In a study aimed to investigate the prognostic impact of CCND1 overexpression in patients with resectable NSCLC, a relationship has been found between CCND1 overexpression and prognostic factors, such as T status, N status and stage[20]. However, prognostic value of CCND1 overexpression in NSCLC patients could not be demonstrated in another study[21]. Although evaluation of prognostic effect was not aimed in our study, but we found that AA + AG type CCND1 gene polymorphism was significantly higher in patients with metastatic disease. Thus, possibly it might negatively affect survival in patients with lung cancer.

In conclusion, despite limitations in our study, such as small number of patients and smoking status of patients, our findings support the role of CCND1 gene polymorphism in lung cancer and development of metastasis, resulting in poor survival. Thus, CCND1 gene polymorphism may be a predictor for detecting patients with poor survival who have metastatic disease. However, to confirm this, further studies are needed with a greater number of patients.

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