

## RESEARCH ARTICLE

# Association of the Cylin D1 G870A Polymorphism with Laryngeal Cancer: Are they Really Related?

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### Abstract

**Background:** Cylin D1 (CCDN1) is an important regulator of the cell cycle whose alterations are thought to be involved in cancer development. There have been many studies indicating CCDN1 amplification or over-expression in a variety of cancer types. In addition to gene amplification, the G870A polymorphism may be related with altered CCDN1 activity, and therefore with cancer development. This hypothesis has been tested in different cancer types but results have been contradictory. We therefore aimed to investigate any relationship between CCDN1 A870G genotypes and laryngeal squamous cell cancer development and progression. **Materials and Methods:** A total of 68 Turkish patients with primary laryngeal squamous cell cancer and 133 healthy controls were enrolled. Polymerase chain reaction-restriction fragment length polymorphism analysis was used to determine the CCDN1 genotypes. **Results:** No significant association was detected between CCDN1 genotypes and laryngeal squamous cell cancer (LxSCCa) development. Similarly CCDN1 genotypes were not related to clinical parameters of Lx SCCa. However, there was a very significant association between CCDN1 G allele and presence of perineural invasion ( $p=0.003$ ; OR: 1.464; CI% 1.073-1.999). CCDN1 G allele frequency was significantly higher in the individuals with perineural invasion (85.7%) when compared to those without (58.5%). The 2 patients who died of disease were both found to possess the GG genotype. **Conclusions:** These results pose a controversy in suggesting a protective role of the G allele against LxSCCa development and support the association of CCDN1 gene GG genotype with mortality in patients with LxSCCa.

**Keywords:** SNP - CCDN1 - A870G - pro241pro - laryngeal squamous cell cancer - Turkey

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### Introduction

Laryngeal cancer is a multifactorial disease, such as other cancer types and is generated by both genetic and environmental factors. Even though laryngeal cancers are not one of the most common seen cancers world-wide, their incidences are reported to be 8% of all tumors. They constitute about 30% of all malignant tumors in the head and neck area and represent a relatively low-survival rate. Tobacco smoking and consumption of alcoholic beverages are well-known risk factors for these tumors (Cann et al., 1985; Koufman et al., 1998; Landis et al., 1998; Berrino et al., 1999; Boffetta et al., 2001; Nilsson et al., 2005; Tuncer et al., 2009).

Since the mortality of cancer is mostly due to late detection of the disease, more specific markers are needed for early diagnosis. In this respect, common molecular alterations considered to be potential markers of tumors have been "top subjects" of cancer researches. As one of these candidate markers; rearrangement of 11q13 region, which particularly leads to amplification of CyclinD1

(CCDN1), is commonly observed in laryngeal cancers (Callender et al., 1994; Jares et al., 1994; Bellacosa et al., 1996; Akervall et al., 1997; Rydzanicz et al., 2006).

CCDN1 is a key gene in cell cycle regulation which controls the G1-S transition during cell cycle. High activity of CCDN1 causes premature cell passage through G1 check-point which results in accumulation of DNA damage and finally in abnormal cell proliferation (Hall et al., 1996; Pabalan et al., 2008).

In accordance with this critical function, amplification of this key regulator has been observed in a variety of cancer types including breast, ovarian, bladder, esophageal, gallbladder, stomach, laryngeal, lung and liver cancers. The amplification of CCDN1 was correlated with poor prognosis, distant metastasis and frequent recurrences in laryngeal cancers (Motokura et al., 1993; Jares et al., 1994; Michalides et al., 1995; Sherr et al., 1995; Naitoh et al., 1995; Betticher et al., 1996; Palmero et al., 1996; Hibberts et al., 1999; Knudsen et al., 2006).

In addition to the known amplification mechanism, a specific variant of CCDN1 generates an alternative

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transcript with a prolonged half-life, which eventually means: increased number of active CCND1. This specific variant creates a guanine adenine transition at codon 242 on exon 4 which inhibits an amino acid substitution (pro241pro) yet production of a truncated splice variant, called transcript b, without PEST motif containing exon 5. Since PEST domain is critical for the degradation of CCND1, transcript b (870A allele) has been shown to have a longer half-life than its wild form transcript a (G870) Betticher et al. (1995). Thus, individuals with CCND1 870A genotype are suggested to more easily bypass the G1-S check point and contribute to cancer development (Weinstein et al., 1997; Sawa et al., 1998; Solomon et al., 2003; Schernhammer et al., 2006; Zhang et al., 2011).

The possible relation between G870A polymorphism and cancer development have been examined in a number of cancer types and ended up with variable results. Association of this specific variant with cancers such as, colorectal Zhang et al.(2011), lung Qiuling et al. (2003), esophageal (Wang et al., 2003; Zhang et al., 2003), breast (Grieu et al., 2003; Krippel et al., 2003; Shu et al., 2005), oral Huang et al. (2012) and squamous cell carcinoma of head and neck Zheng et al. (2001) has been reported in a number of studies including meta-analysis reports. However, some studies indicated no or weak association with CCND1 870A genotype and cancer risk, specifically for esophageal (Jain et al., 2007; Zhuo et al., 2012), breast and colorectal Grieu et al. (2003) carcinoma.

Although the effects of CCND1 polymorphism have been tested in various cancer types, there are only a limited number of studies that subjected directly to laryngeal squamous cell cancer since this disease is mostly investigated within the head and neck cancer group. Studies that have been presented so far indicate contradictory results. CCND1 GG genotype was reported to be associated with poor differentiation and shortened time for tumor recurrence in squamous cell cancers (SCC) of head and neck Matthias et al. (1998). Another study has shown the association of this genotype with longer disease-free survival and reduced incidence of local relapse in non-small cell lung cancer Betticher et al. (1995).

In a more recent study, GG genotype was suggested as “protective” variant which decreases the risk of larynx cancer development since it was found more frequently in the control group Rydzanicz et al. (2006).

On the other hand, CCND1 AA genotype was suggested as a risk factor, especially between nonalcoholic, for squamous cell carcinoma of the upper aero digestive system Nishimoto et al. (2004).

Considering the present contradiction between represented results, the aim of this study was to establish the possible relation between CCND1 alleles and genotypes and larynx cancer in a group of 65 Turkish patients with Laryngeal SCC and 83 healthy controls.

## Materials and Methods

### Study participants

Patients were selected among the individuals who were followed up in ENT clinic for three years. Regarding

to low frequency of the disease in female individuals (only three), CCND1 G870A gene polymorphisms were investigated in 65 male patients with Lx SCC and 83 age matched male healthy control subjects. The study was approved by the Institutional local Ethics Committee with the study ID: B.02.1.VGM.4.00.01 in accordance with the Declaration of Helsinki revised in 2004. Informed consent was obtained from each patient.

The mean ages of patients and control group were 61.59±8.9 and 56.3±12.6 years, respectively. Patients' questionnaires, pathology records and laryngoscopy findings were collected from the medical charts of the patients to confirm the diagnosis and cancer staging. The control subjects, who were not taking any regular medication by that time, were randomly selected among healthy volunteers. The blood samples were collected after pathological diagnosis prior to any surgical, chemotherapeutic or radiation therapy from those who had not undergone blood transfusion.

Clinical and pathological informations on all larynx SCC diagnoses were confirmed by manual review of the pathology reports and endoscopic findings of Otorhinolaryngology Department. Stage of the laryngeal cancers were defined according to the American Joint Committee on Cancer (AJCC) TNM classification. Glottic and supraglottic tumors were categorized in T1, T2, T3 and T4 subclasses according to the localization of the tumor. Nodal status was categorized as no regional lymph nodes affected (N0), metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension (N1) or metastasis in a single ipsilateral lymph node, >3 cm but ≤6 cm in greatest dimension or multiple lymph nodes ipsi or contralaterally (N2a,b,c).

Genomic DNA was extracted from peripheral whole blood containing EDTA according to salting-out technique. DNA was isolated from the blood leukocytes in 10 ml EDTA by the method of Miller et al. (1988) based on sodium dodecyl sulphate lysis, ammonium acetate extraction, and ethanol precipitation Miller et al. (1988).

### Polymerase chain reaction (PCR) for CCND1 gene

Template DNA (0.5-1.0 µg) was used in a PCR under sterile conditions. A concentration of 0.4 µmol/l of each primer was used for the reaction. The forward primer was 5'GTGAAGTTCATTTCCAATCCGC-3' and the reverse primer was 5'GGGACATCACCCCTCACTTAC-3' in a volume of 25 µl containing 1.5 mM MgCl<sub>2</sub>, 50mM KCl, 10 mM Tris-HCl (pH 8.4), 0.16 mM each of deoxynucleotide triphosphate (MBI Fermentas, Vilnius, Lithuania), and 1 unit of Taq polymerase (MBI Fermentas, Vilnius, Lithuania). The reaction mixture was initially denatured at 94°C for 5 minutes, followed by 35cycles 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. The PCR product exhibited a 167 base pair fragment. PCR products (10 ml) were digested with 15U NciI (MBI Fermentas) at 37°C for 3 hours, and visualized by electrophoresis on 3% agarose containing 0.5 mg/ml ethidium bromide. The 167 bp PCR product generated is not cut by NciI if the

A allele is present, whereas the product from the G allele is cut to produce fragments of 145 and 22 bp. CCND1 G870A polymorphism was typed by visualization under ultraviolet light and photographing with a Polaroid camera. The CCND1 G870A alleles were identified in each sample. The allele types were determined as follows: a single 167 bp fragment for the AA genotype, two fragments of 22 and 145 bp for the GG genotype, and three fragments of 22, 145 and 167 bp for the AG genotype.

#### Statistical analysis

All statistical analyses were carried out using SPSS version 7.5 for Windows (SPSS Inc, Chicago, USA). Numeric values were analyzed by Student's t-test. Differences in characteristics between patients with laryngeal cancer and controls, as well as disparities in genotype and allele frequencies, were assessed with the chi-square test. CCND1 G870A allele frequencies were estimated by gene counting methods. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated to estimate the risk for larynx cancer. The threshold for significance was  $p < 0.05$ .

## Results

65 male patients with Lx SCC and 83 male healthy controls were included in the study. Genotype and allele frequencies of patients and controls are detailed in Table 1. No statistically significant difference was found between study and control groups with regard to genotypes and allele distribution. Likewise there was no significant difference in Lx SCC patients with regard to CCND1 genotypes and alleles A and G. Genotype distributions for CCND1 A870G polymorphism were in agreement with Hardy-Weinberg equilibrium both in patient and control groups ( $p = 0.753$ ,  $p = 0.796$  respectively).

Clinicopathological features of patients according to CCND1 genotypes are presented in Table 2. No statistically significant association was found between CCND1 genotypes and presence of reflux, alcohol consumption, tumor T stage, lymph node invasion, tumor differentiation and tumor site in Lx SCC patients (Table 2). Smoking and alcohol consumption were detected in 63 (96.9%) and 35 (54.7%) patients respectively.

A slight relation was found between the homozygote AA/GG genotype and advanced tumor stage (T3, T4), poor differentiation and nodal metastasis in the patient group. However, the difference was not statistically significant ( $p > 0.05$ ).

Glottic tumors were more frequently seen in patients

**Table 1. Genotype and Allele Frequencies of Study Group and Controls**

SNP	Controls		Larynx		Chi-s quare	p
	Cases	Frequency	Cases	Frequency		
	(n=65)		(n=65)			
Genotype	GG	23 27.7	12	18.5	1.74	0.417
	AG	37 44.6	32	49.2		
	AA	23 27.7	21	32.3		
Alleles	A	83 50	74	56.9	1.4	0.236
	G	83 50	56	43.1		

**Table 2. Clinicopathological Features of Patients According to CCND1 Genotypes**

		Larynx Cancer		
		GG (%)	AG (%)	AA (%)
Diferentiation	Poor	2(22.2)	3(33.3)	4(44.4)
	Medium	7(15.9)	23(52.3)	14(31.8)
	Well	3(27.3)	5(45.5)	3(27.3)
Tumor location	Glottic	6(15.0)	20(50.0)	14(35.0)
	Supraglottic	5(22.7)	11(50.0)	6(27.3)
Reflux	Yes	5(17.2)	15(51.7)	10(31.0)
	No	7(20.0)	16(45.7)	12(34.3)
Tumor recurrence	Yes	1(11.1)	7(77.8)	1(11.1)
	No	11(20.0)	24(43.6)	20(36.4)
Muscle invasion	Yes	7(15.9)	21(47.7)	16(36.4)
	No	4(22.2)	10(55.6)	4(22.2)
Tumor stage	T1	0 (0)	4(57.1)	3(42.9)
	T2	2(18.2)	5(45.5)	4(36.4)
	T3	7(18.9)	17(45.9)	13(35.1)
	T4	3(33.3)	5(55.6)	1(11.1)
Metastasis	Yes	2(11.1)	12(66.7)	4(22.2)
	No	10(21.7)	19(41.3)	17(37.0)
Lymph node	N0	6(15.8)	16(42.1)	16(42.1)
	N1	5(22.7)	14(63.6)	3(13.6)
	N2	1(25.0)	1(25.0)	2(50.0)
Perineural invasion	Yes	5(23.8)	13(61.9)	3(14.3)
	No	7(16.3)	18(41.9)	18(41.9)
Smoking status	Ever smokers	12(19.0)	30(47.6)	21(33.3)
	Never smokers	0 (0)	2(100)	0(0)
Alcohol consumption	Yes	8(22.9)	18(51.4)	9(25.7)
	No	4(13.8)	13(44.8)	12(41.4)

with AA genotype or A allele while supraglottic tumors were more frequent in GG genotype or G allele however the difference was not statistically significant.

There was a very significant association between CCND1 G allele and presence of perineural invasion ( $p = 0.003$ ; OR: 1.464; CI% 1.073-1.999). CCND1 G allele frequency was significantly higher in the individuals with perineural invasion (85.7%) when compared to those without (58.5%). The 2 patients who died of disease were both found to possess GG genotype .

## Discussion

With the knowledge that we have accrued for years, now it is clear that alterations on key regulators of cell cycle mechanisms are involved in carcinogenesis. CCND1 gene is one of these important regulators of cell cycle and known to be overexpressed in numerous cancer types (Motokura et al., 1993; Michalides et al., 1995; Naitoh, 1995; Sherr, 1995; Betticher, 1996; Hall et al., 1996; Palmero et al., 1996; Hibberts et al., 1999; Knudsen et al., 2006; Pabalan et al., 2008). This observed importance of CCND1 up regulation in carcinogenesis is due to its critical function on G1-S transition during the cell cycle. Cyclin D1 is a product of CCND1, and forms complexes with Cyclin dependent kinases (CDKs) Cdk4 and Cdk6 which control the G1-S transition. Furthermore, Cyclin D1 catalyzes the phosphorylation of retinoblastoma (Rb) protein which means the disassociation of transcription factor E2F from Rb and eventually the transcription of "cell division" genes (Hunter et al., 1994; Sherr, 1996; Muller et al., 1997; Sawa et al., 1998; Matthias et al., 1999; Gleich et al., 2002; Nishimoto et al., 2004).

In addition to alterations in gene expression levels,

a specific polymorphism, CCDN1 G870A, is shown to cause production of an alternative transcript with longer half-life which increases Cyclin D1 levels in cell. Thus, individuals with CCDN1 870A allele are suggested to more easily bypass the G1-S check point and develop cancer when compared to the ones with wild type CCDN1 G870 (Betticher et al., 1995; Solomon et al., 2003).

This hypothesis has been tested in numerous studies however findings are contradictory. Some of the researches suggested the association of CCDN1 G870A with cancer development risk, prognosis and survival in non-small lung cancers Qiuling et al. (2003), breast cancer (Griew et al., 2003; Krippel et al., 2003; Shu et al., 2005; Yaylim et al., 2009), head and neck cancer Zheng et al. (2001), ovarian cancer Dhar et al. (1999), pituitary adenomas Simpson et al. (2001) and larynx and oral cavity Izzo et al. (2003). On the other hand, no significant association between G870A polymorphism and bladder cancer Sanyal et al. (2004), pituitary adenomas Sanyal et al. (2007) was reported. Regarding Lx SC cancers, results are in harmony with other cancer types. CCDN1 A allele was associated with increased CCDN1 expression and poor disease outcome in advanced T stages of the larynx and/or oral cavity in a study of 31 patients (Izzo et al., 2003; Shu et al., 2005).

In another study with 66 laryngeal squamous cell carcinoma patients Monteiro et al. (2004), no significant association was observed between CCDN1 A870G genotypes and disease stage, tumor differentiation grade or recurrent disease but a significant association was detected between CCDN1 A870G polymorphism and both disease-free and overall survival in accordance with previous findings of Matthias et al. (1998).

The wild type GG genotype was suggested to be the "protective variant" for larynx cancer by Rydzanicz et al. (2006) and AA genotype was suggested to be a risk factor for head and neck squamous cell cancer Zheng et al. (2001).

In a study with upper aero digestive tract cancers, including larynx, higher cancer progression rate was reported in individuals with A allele (both AA and AG) when compared to subjects with GG genotype and CCDN1 genotype and protein expression was suggested to be important risk markers for laryngeal cancer (Izzo et al., 2004; Papadimitrakopoulou et al., 2009).

According to our results, no significant association was detected between CCDN1 genotypes and laryngeal squamous cell cancer development, tumor T stages, localization, histological tumor differentiation, presence of reflux. However, we detected a significant association between CCDN1 G allele and presence of perineural invasion. CCDN1 G allele frequency was significantly higher in the individuals with perineural invasion (85.7%) when compared to those without (58.5%) ( $p=0.003$ ; OR: 1.464; CI% 1.073-1.999).

Cyclin D1 GG870 genotype has been found to be associated with poorly differentiated tumors and with a reduced disease-free interval in SCC of the head and neck Matthias et al. (1998). Later on, in 2004, Monteiro et al. based on the results derived from their study on CCND1 A870G polymorphism supported the observation that

GG870 genotype was associated with a shorter disease free interval and a reduced overall survival in laryngeal cancer patients Monteiro et al. (2004). Regarding our results that demonstrated the association of GG genotype with perineural invasion which is a well-known poor prognostic indicator for Lx SCC we may suggest in line with Yilmaz et al. (1998) that the GG genotype may worsen the disease prognosis Yilmaz et al. (1998). Indeed the 2 patients who died of disease were both found to possess GG genotype in the present study. The association of mortality with GG genotype was also in agreement with the findings of above mentioned authors (Matthias et al., 1998; Monteiro et al., 2004).

Among the 18 patients with distant metastasis, 12 (66.7%) had AG genotype and the difference was close to statistical significance ( $p=0.068$ ; OR: 1.614; CI% 1.004-2.595). Other interesting findings that were also very close to statistical significance were presence of the AG genotype in the 78% of the patients who had tumor recurrence ( $p=0.057$ ; OR: 1.782; CI% 1.125-2.825) and the high frequency of the G allele (80.8%) in the patients with nodal metastasis ( $p=0.056$ ; OR: 1.395; CI% 1.003-1.940). These results in the AG heterozygote individuals may support the above mentioned finding of the relation between CCDN1 G genotypes and the disease progression.

Another interesting finding was the relation between genotypes and tumor localization. AA genotype or A allele was more frequently seen in tumor with glottis localization and GG genotype or G allele was mostly seen in supraglottic tumors. Since supraglottic tumors are suggested to more likely to spread and metastasize, (Coleman et al., 1993) these findings were inconsistent with the "protective" features of CCDN1 GG genotype put forward by Izzo and Papadimitrakopoulou (Izzo et al., 2004; Papadimitrakopoulou et al., 2009).

Limitations of the present study were its small sample size that interfered with more accurate definition of Cyclin D1 genotypes and the restriction of the control group to non-smokers and non-alcoholic individuals. Although, smoking and alcohol consumption are well established risk factors for larynx SCC development; we were not able to verify the correlation between genotypes and tobacco and alcohol use. Difficulty of generating a homogenized study group for larynx patients, particularly for the gender, restricted the patients only to males and limited the number of individuals that were investigated within this study.

We examined in this study the relation of CCDN1 A870G polymorphism with larynx SCC development risk. No statistically significant difference was found between study and control groups with regard to genotype and allele distribution. Likewise there was no significant difference in Lx SCC patients with regard to CCDN1 genotypes and alleles A and G. G allele was significantly higher in patients with perineural invasion. These findings are needed to be verified by further investigations and with a larger sample size including both male and female individuals. Nonetheless, these results contribute to existing data on larynx cancer investigations and represent some more possible contradictions to be explained in further studies.

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