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Cyclin D1 polymorphism and risk for squamous cell carcinoma of the head and neck: a case–control study

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A G→A polymorphism (G870A) in exon 4 of the cyclin D1 (CCND1) gene creates an alternative splice site in its mRNA, encoding a protein with an altered C-terminal domain. It has been suggested that DNA damage in cells with the A allele bypasses the G1/S checkpoint of the cell cycle more easily than damage in cells without the A allele. Because CCND1 plays a critical role in cell cycle control and reduced DNA repair capacity is associated with an increased risk for squamous cell carcinoma of the head and neck (SCCHN), we hypothesize that this CCND1 polymorphism modulates individual susceptibility to SCCHN. To test this hypothesis we evaluated the frequency of the polymorphism in a hospital-based case–control study of 233 newly diagnosed SCCHN patients and 248 non-cancer controls. The cases and controls were frequency matched by age (±5 years), sex and tobacco use. All subjects were non-Hispanic whites. We found that the A allele frequency was slightly higher in the cases (0.485) than in the controls (0.425), but the difference was borderline statistically significant (P = 0.064). The frequencies of the CCND1 AA, GA and GG genotypes were 23.6, 49.8 and 26.6%, respectively, in cases and 16.5, 52.5 and 31.5%, respectively, in controls. Multivariate logistic regression analysis adjusting for age (in years), sex, smoking and alcohol use was performed to calculate odds ratios (OR) and 95% confidence intervals (CI). Compared with the wild-type CCND1 GG, the CCND1 A G genotype was associated with a non-significantly increased risk (adjusted OR 1.15, 95% CI 0.75–1.76), but the CCND1 AA genotype was associated with a significantly increased risk (adjusted OR 1.77, 95% CI 1.04–3.02) for SCCHN. Results from a trend test using a logistic regression model were statistically significant (P = 0.004). Among the cases the mean age of onset was 59.0, 56.8 and 55.5 years for the GG, GA and AA genotypes, respectively. In the stratification analysis the CCND1 AA variant genotype was associated with a ≥3-fold increased risk in individuals who were <50 years old (OR 3.18, 95% CI 1.19–8.46), females (3.57, 1.26–10.0), non-smokers (3.71, 1.37–10.1) and non-alcohol users (4.76, 1.61–14.0). These results suggest that the CCND1 polymorphism is associated with early onset of SCCHN and contributes to susceptibility to SCCHN in this population.

Introduction

In 2001, an estimated 40,100 new cases of squamous cell carcinoma of the head and neck (SCCHN) will occur and 11,800 deaths will be caused by this disease in the USA (1). Epidemiological studies show that both tobacco use and alcohol consumption are the major risk factors for SCCHN (2). However, only a small fraction of smokers or alcohol users develop SCCHN, suggesting that genetic susceptibility and interactions between genetic and environmental factors play important roles in the etiology of SCCHN (3).

Because carcinogenesis of the head and neck involves abnormalities in carcinogen metabolism, DNA repair and cell cycle control or apoptosis (4–7), polymorphisms of genes involved in these pathogenic pathways are good candidates for investigations of genetic susceptibility. Normal cell cycle control ensures a rest in the cell cycle allowing DNA damage in a cell to be repaired before the cell begins the process of growth, mitosis and division (8–10). The transition through G1 to S phase of the cell cycle is regulated by cyclin-dependent kinases (CDKs) (8). Cyclin D1 (CCND1) is a key regulatory protein in this process, playing a critical role in the transition from G1 to S phase of the cell cycle (9). The activity of CCND1 reaches a maximum during G1 phase; and is associated with CDK4 and CDK6 in mid to late G1 phase, therefore, it is one of the major cyclins involved in this transition (8). Alterations in CCND1 are thought to be involved in carcinogenesis because activation of CCND1 and overexpression of CCND1 have been found in a variety of tumors, including those of the breast, head and neck, esophagus, larynx and lung (11–13).

Betticher and colleagues (14) identified a single base pair polymorphism (G870A) in CCND1. This polymorphism does not lead to an amino acid change, but the variant nucleotide with the A allele bypasses the G1/S checkpoint of the cell cycle control mechanism more easily than damage in cells not carrying the polymorphism (15).

Because CCND1 plays a critical role in cell cycle control and there is evidence that reduced DNA repair capacity is associated with increased risk of SCCHN (16), we hypothesize that the G→A CCND1 polymorphism may modulate individual susceptibility to SCCHN. To test this hypothesis we conducted a hospital-based case–control study of 233 newly diagnosed SCCHN patients and 248 non-cancer controls.

Materials and methods

Study subjects

From May 1995 to October 2000 patients with histopathologically confirmed SCCHN (incident cases involving only primary tumors of the oral cavity,
Genotyping
From each blood sample, a leukocyte cell pellet was obtained from the buffy coat by centrifugation of 1 ml of whole blood. The cell pellet was used for DNA extraction. The Qiagen DNA Blood Mini Kit (Qiagen, Valencia, CA) was used according to the manufacturer’s instructions to obtain genomic DNA. The DNA purity and concentration were determined by spectrophotometric measurement of absorbance at 260 and 280 nm.

PCR—single-strand conformation polymorphism (SSCP) analysis was used to genotype the CCND1 polymorphism in exon 4. For PCR the primers were 5'-TACATCGCCCTACACGCTTCC-3' (sense) and 5'-TTG 2 GCAC-CAGGCTCCGATCTTCC-3' (antisense), which generate a 138 bp fragment. These fragments were amplified in 25 μl of reaction mixture containing ~50 ng genomic DNA, 6.25 pmol each primer, 1× PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0 at 25°C, and 0.1% Triton X-100), 1.5 mM MgCl2, 0.2 mM each dATP, dTTP and dGTP, 0.1 mM dCTP, 1 μCi [3P]dCTP and 1 U Taq polymerase (Promega, Madison, WI). The PCRs were performed by incubating the fragments and reaction mixture at 95°C for 5 min, subjecting them to 26 cycles of 94°C for 30 s and 65°C for 30 s and incubating them at 72°C for 1 min, with a final elongation step at 72°C for 10 min. For SSCP analysis, 4 μl of PCR product was mixed with 4 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue). The mixture was denatured at 95°C for 5 min and then immediately put on ice. Four microlitres of the mixture was loaded on a mutation detection enhancement gel (FMC BioProducts, Rockland, ME) for electrophoresis at 35 W for 4 h. After electrophoresis the gels were dried and respectively, in controls (Table II). Although the CCND1 genotype was more common in cases than in controls, the difference was only borderline statistically significant (P = 0.064). The observed distribution of CCND1 genotypes in controls (GG:GA:AA, 78:129:41) was not statistically different from that (82:118:48) expected from the Hardy–Weinberg equilibrium equation (P = 0.565). The frequencies of the CCND1 AA, GA and GG genotypes were 23.6, 49.8 and 26.6%, respectively, in cases and 16.5, 52.0 and 31.5%, respectively, in controls (Table II). Although the CCND1 AA genotype was more frequent in cases than in controls, the difference in genotype distribution was not statistically significant (P = 0.129).

Statistical Analysis
Univariate analysis was first performed to calculate the frequency of each genotype. The observed genotype frequencies were compared with those calculated from Hardy–Weinberg equilibrium theory (r2 = 2pq + q2, where r is the frequency of the variant allele and q = 1 – p). Subjects who had smoked >100 cigarettes in their lifetime were defined as ever smokers; in this group those who had reported quitting at least 1 year before interview (controls) or diagnosis (cases) were defined as former smokers. Subjects who had reported drinking alcoholic beverages at least once a week >1 year previously (before diagnosis or interview) were defined as ever drinkers; in this group those who had quit drinking >1 year before diagnosis were defined as former drinkers and the others as current drinkers. The odds ratios (ORs) and their 95% confidence intervals (CIs) for the CCND1 genotype were calculated by logistic regression analysis, with adjustment for age (in years), sex, smoking status and alcohol use. For logistic regression analysis the

![Figure 1](image)

**Table I.** Distribution of selected demographic variables in SCCHN patients and controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n = 233)</th>
<th>Controls (n = 248)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤45</td>
<td>39</td>
<td>48</td>
<td>0.129</td>
</tr>
<tr>
<td>46–55</td>
<td>70</td>
<td>77</td>
<td>0.311</td>
</tr>
<tr>
<td>56–65</td>
<td>57</td>
<td>67</td>
<td>0.270</td>
</tr>
<tr>
<td>&gt;65</td>
<td>67</td>
<td>56</td>
<td>0.226</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>169</td>
<td>173</td>
<td>0.698</td>
</tr>
<tr>
<td>Female</td>
<td>64</td>
<td>75</td>
<td>0.302</td>
</tr>
<tr>
<td>Smoking status</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>65</td>
<td>85</td>
<td>0.343</td>
</tr>
<tr>
<td>Current</td>
<td>102</td>
<td>101</td>
<td>0.407</td>
</tr>
<tr>
<td>Alcohol use</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>73</td>
<td>86</td>
<td>0.346</td>
</tr>
<tr>
<td>Former</td>
<td>50</td>
<td>68</td>
<td>0.274</td>
</tr>
</tbody>
</table>

*Two-sided χ2 test.

CCND1 genotype was recorded as a dummy variable. For stratification analysis by age, cases aged 50 or younger were considered as having an early age of onset. A trend test for the number of A alleles was also performed using a logistic regression model. All the statistical analyses were performed using Statistical Analysis System software V6.12 (SAS Institute, Cary, NC).

Results
The analysis included 233 cases and 248 controls, all of whom were non-Hispanic whites. As shown in Table I, the cases and controls were adequately matched by age, sex and smoking status. However, alcohol use, which was not a matching variable, was significantly more common in cases than in controls (P < 0.001). The distributions of CCND1 genotype and allele frequencies in SCCHN patients and controls are shown in Table II. The A allele frequency was slightly higher in cases (0.485) than in controls (0.425), but this difference was only borderline statistically significant (P = 0.064). The observed distribution of CCND1 genotypes in controls (GG:GA:AA, 78:129:41) was not statistically different from that (82:118:48) expected from the Hardy–Weinberg equilibrium equation (P = 0.565). The frequencies of the CCND1 AA, GA and GG genotypes were 23.6, 49.8 and 26.6%, respectively, in cases and 16.5, 52.0 and 31.5%, respectively, in controls (Table II). Although the CCND1 AA genotype was more frequent in cases than in controls, the difference in genotype distribution was not statistically significant (P = 0.129).

To evaluate the association between the CCND1 polymorphism and risk of SCCHN, crude and adjusted ORs and their 95% CIs were calculated (Table II). Compared with the wild-type CCND1 GG, the CCND1 AA genotype was associated with a significantly increased risk of SCCHN (adjusted OR 1.77, 95% CI 1.04–3.02). The adjusted OR for the CCND1 A G genotype was 1.15 (95% CI 0.75–1.76). The trend test using a logistic regression model showed that the effects of genotype on the OR were statistically significant (P = 0.044) after adjustment for age (in years), sex, smoking status and alcohol use (Table II).

Table III shows the CCND1 genotype frequencies in subjects stratified by age, sex and smoking and alcohol status. The
mean ages of cancer onset for patients with the GG, GA and AA genotypes were 59.0, 56.8 and 55.5 years, respectively, but the differences were not significant (data not shown). However, in the younger subgroup (age ≤ 50 years) the OR was 3.18 (95% CI 1.19–8.46) for the AA genotype after adjustment for sex, smoking and alcohol status. Females exhibited an elevated risk (3.57, 95% CI 1.26–10.0) associated with the AA genotype, but this was not evident for men (OR 2.56). In addition, significantly elevated ORs associated with the AA genotype were evident in those individuals who were younger, male, non-smokers and non-alcohol users. In particular, this variant genotype is associated with an early age of SCCHN onset. These results are consistent with the notion that the CCND1 AA genotype is a genetic susceptibility factor in SCCHN.

We have previously reported an association between reduced DNA repair capacity and risk of SCCHN (16). Cell cycle control is central to repair of DNA damage before cell division and CCND1 controls the transition from G1 to S phase. The CCND1 gene is located at 11q13 and can act as an oncogene (17–19); it is often overexpressed in tumor cells. In cultured cells a cDNA clone of the CCND1 gene contributes to cell transformation by complementing a defective adenovirus E1A oncogene. Rimokh et al. (20) showed that rearrangement and overexpression of CCND1 (BCL1/PRAD1) are consistent features of t(11q13)-bearing B lymphoid tumors (particularly mantle cell lymphoma). Its deregulation is thought to perturb the G1/S transition of the cell cycle and thereby to contribute to tumor development. As suggested by previously published studies, rearrangement of the 3’-untranslated region of CCND1 may contribute to its activation in some lymphoid tumors. Moreover, the finding that the half-life of CCND1 mRNA was >3 h in three cell lines (it is only 0.5 h in normal tissues) stresses the importance of post-transcriptional derangement in the activation of CCND1 (20).

As one of the major genes controlling the cell cycle transition from G1 to S phase, abnormal CCND1 may lead to an abnormal cell cycle. Overexpression of CCND1 may lead to premature cell passage through the G1/S transition, resulting in propagation of unrepaired DNA damage, accumulation of genetic errors and a selective growth advantage for altered cells. CCND1 overexpression also appears to alter tumor cell sensitivity to ionizing radiation (21). In tumor tissues CCND1 is
often overexpressed at both the transcriptional and translational levels (22) and its amplification or overexpression is associated with a more rapid and frequent recurrence of cancer (23–25). Marchetti et al. (26) reported that a high frequency of CCND1 amplification/overexpression is one of the important steps in lung cancer development. Overexpression and/or amplification of CCND1 are common alterations in SCCHN (27) and may be associated with survival of such patients (28).

A single base pair polymorphism (exon 4, G870A) in the CCND1 gene affects gene splicing (14). Previous studies showed that frequency of the AA genotype was higher in breast cancer (43%) and parathyroid adenomas (32%) than in controls (21%) (29). Recently the A allele of CCND1 was shown to be associated with an early age at onset of hereditary non-polyposis colorectal cancer, acting as a risk modifying gene (15). Furthermore, the CCND1 genotype has also been associated with clinical outcome in small cell lung cancer and SCCHN patients (14).

In the present study we found that the CCND1 AA genotype is associated with a relatively high risk of SCCHN and that this risk is markedly increased in subjects who are young, female, non-smoking or non-alcohol using. This finding suggests that the young female non-smoker/non-drinker with SCCHN is part of a unique sub-group of patients and is consistent with the clinical finding that younger patients are more commonly female and are not smokers or drinkers (30–33). When using the GG homozygotes as the reference we found a trend of increased risk for SCCHN associated with the GA heterozygotes and AA homozygotes. Our finding of the AA genotype as a potential risk for an early age of SCCHN onset is also consistent with an early finding in non-hereditary polyposis colorectal cancer (15), in subjects with the AA and GA genotypes tended to develop colorectal carcinoma at an earlier age than those with the GG genotype. Two studies (34,35) have not found the CCND1 genotype to be associated with cancer susceptibility; however, important environmental variables, such as smoking and alcohol consumption, and age were not well matched in either study, which may contribute to the discrepant findings. Although the underlying molecular mechanism for an association between CCND1 genotype and cancer risk is not clear, it is hypothesized that if DNA is damaged and remains unrepaird the higher level of CCND1 protein in cells with the A G or AA genotypes might allow the defective cells to pass through the G1/S phase checkpoint more easily than cells with the GG genotype.

In conclusion, we found that CCND1 polymorphism may contribute to the susceptibility to SCCHN in this non-Hispanic white population, particularly in those who were young, females, non-smokers and non-alcohol users. However, it is likely that our results could be due to chance in the stratification analysis, which may lead to multiple comparisons. Bias may also arise from the limited numbers in the sub-groups analyzed and the possible selection bias inherent in a hospital-based case–control study. Therefore, these results remain preliminary and need to be validated in larger prospective studies.

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References
CCND1 polymorphism and SCCHN


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