A Genotype-Phenotype Examination of Cyclin D1 on Risk and Outcome of Squamous Cell Carcinoma of the Head and Neck


Abstract Purpose: The variant allele of CCND1 G870A encodes a splice variant of the cyclin D1 protein, which possesses an increased half-life. To confirm the phenotypic effect of the variant allele, we examined the immunohistochemical staining pattern of the protein in tumors from a case population of head and neck squamous cell carcinoma (HNSCC) and compared it with the genotype of these individuals. We also examined how this genotype was associated with the risk of HNSCC and if this genotype-phenotype association was related to patient outcome.

Experimental Design: In a population-based case-control study of 698 cases and 777 controls, we both genotyped all participants for the CCND1 gene and did immunohistochemical staining of the cyclin D1 protein in the HNSCC tumors.

Results: The variant AA genotype was significantly associated with positive immunohistochemical staining (P < 0.02), and this variant genotype was associated with a significantly elevated odds ratio of 1.5 (95% confidence interval, 1.1-2.0) for HNSCC overall, with risk greatest in oral and laryngeal sites. Positive immunohistochemical staining was inversely related to human papillomavirus 16 DNA present in the tumor (P < 0.03). The AA genotype and superpositive immunohistochemical staining for cyclin D1 also had independent and significant effects on patient survival.

Conclusions: These results strongly suggest that this splice variant, when present in two copies, is a significant predictor of both the occurrence of HNSCC as well as patient survival after treatment. These data further indicate that this variant protein is an important determinant of individual response to therapy for this disease.

In 2007, almost 46,000 new cases of head and neck squamous cell carcinoma (HNSCC) will be diagnosed, with greater than 11,000 deaths resulting from this disease (1). Tobacco and alcohol use are independently as well as synergistically associated with the incidence of this disease (2, 3), as is human papillomavirus (HPV) infection (4-6). The contribution of genetic variation to HNSCC etiology is an area of intense study, as only a small fraction of those exposed to these agents develop the disease. The precise genes and polymorphisms conferring risk or modifying the action of alcohol, tobacco, or HPV are still being elucidated (7, 8).

Promotion of cell division through dysregulation of the cell cycle is considered a fundamental hallmark of cancer (9) and at the center of this cell cycle regulation are the cyclins and cyclin-dependent kinases (10). Cyclin D1 (encoded by CCND1) interacts with cyclin-dependent kinase 4/6 and, in response to mitogenic signals, leads to phosphorylation of pRB, allowing passage through the restriction point and progression through the G1 phase (11). Cyclin D1 is an established oncogene (12), with overexpression observed in several human cancers (13). Overexpression of cyclin D1 has been observed in HNSCC, and use of neutralizing antibodies to cyclin D1 in a HNSCC cell line inhibited cell cycle progression (14). In CCND1, a polymorphism (G870A, rs603965) has been identified that leads to a splicing variation and may alter sequences in the protein responsible for protein turnover, thereby rendering this oncogenic protein a greater half-life (15). The variant A allele is associated with an increased risk for HNSCC in a hospital-based case-control study in the United States (16) as well as with increased risk for oral premalignant lesions (17). Another European group has described the G allele as variant and reported this allele to be associated with risk for HNSCC (18) as well as with poorer patient survival (19).

The focus of this study was to examine, in a population-based case-control study of HNSCC, the role of CCND1 G870A polymorphism on this disease. We examined the association of the polymorphic alleles of this gene with the expression of the protein in individual tumors using immunohistochemical...
methods and with the occurrence of HNSCC. Finally, we investigated the association of these biomarkers with patient outcome, defined as overall patient survival.

Materials and Methods

Study population. The study population has been previously described (20, 21). Briefly, incident cases of HNSCC were identified from nine medical facilities in the Boston metropolitan area, with histologic classification of malignancy reported by pathology of the participating hospitals and confirmed by independent study pathologist. Population-based controls were drawn from the same greater Boston population and matched to cases by gender, age (±3 years), and town of residence using the Massachusetts town lists. All cases and controls enrolled in the study provided written informed consent as approved by the institutional review boards of the participating institutions. Archived pathology specimens were used to construct tissue microarrays for the immunohistochemical analyses. A total of 226 tumors had adequate samples available for tumor array production and subsequent molecular analyses. Survival time was determined for cases using publicly available databases. Data on HPV16 serology in this case-control study as well as HPV16 DNA in case tumor samples have been previously reported (6).

Genotyping. DNA was obtained from whole blood or, where blood was unavailable, exfoliated buccal cells using the QIAmp DNA extraction system (Qiagen). Examination of the CCND1 G870A polymorphism was done using a commercially available primer/probe set (Applied Biosystems) detected using an ABI 3500 Sequence Detection System (Applied Biosystems). Genotyping was done in a blinded fashion, appropriate controls were included in each run, and ~10% of samples were duplicated in a coded fashion as quality assurance with >95% concordance observed between replicates. We have labeled the G allele as the wild-type and A as variant, as the G allele is considered the “ancestral” allele in the dbSNP database.

Immunohistochemistry. Each tumor was arrayed in duplicate on the tissue microarray. Array slides were prepared, stained, and scored as previously described (22–24). Briefly, antigen retrieval was done in a pressure cooker for 40 min total (19 p.s.i./127°C) using a citrate buffer (Biogenex). The tissue was incubated with 1:200 dilution of anti–cyclin D1 antibody against the COOH terminus of human cyclin D1 (Biocare Medical) for 30 min. Primary antibody was detected with peroxidase-conjugated streptavidin and dimethylbenzidine chromogen (Biogenex). Scoring was done in blinded fashion by the study pathologist (C.C.B.), and staining was scored as insufficient or lacking tumor on slice, negative (no staining), equivocal (1-5% cells staining), positive (>5-90% cells staining), and superpositive (>90% cells staining with opaque chromogen). In analyses, samples scoring as negative or equivocal for one or both tumor samples were grouped together, samples with one or both considered positive were counted as positive, and samples where both samples scored as superpositive were scored as superpositive.

Statistical analysis. Data were analyzed using the Statistical Analysis System software, and all P values represent two-sided statistical tests. Tests for Hardy-Weinberg equilibrium were conducted. Unconditional logistic regression was used to evaluate the independent effect of the variant CCND1 G870A polymorphism on HNSCC risk, controlling for the matching factors of age and gender as well as confounders known to be associated with HNSCC risk, including tobacco use (pack-years smoked in quartiles based on distribution in controls), alcohol use (lifetime average drinks per week, in quartiles based on distribution in controls), and HPV16 serology (positive or negative based on titer). For analyses by tumor location, cases were grouped according to International Classification of Diseases 9 (ICD9) code, with oral cancer encompassing ICD9 141-145, pharyngeal cancers 146-149, and laryngeal cancers 161. As homozygous GG and heterozygous G/A genotypes had similar odds ratios (OR), these groups were combined as the referent, and the OR for the association of the homozygous variant (A/A) genotype with disease was determined in all analyses. To evaluate synergistic effects between genotype and these exposures, interaction terms were included and the significance of the interaction was evaluated using the likelihood ratio test.

To examine the relationship between cyclin D immunohistochemical score and CCND1 genotype as well as tumor HPV16 DNA status, 2 × 2 tables were constructed, and differences in the prevalence of genotype or HPV16 DNA status by cyclin D immunohistochemical staining were examined using a Fisher’s exact test. Due to reduced sample size and cells in the table containing no observations, logistic regression analysis could not be done.

Patient survival was first examined using Kaplan-Meier survival probability curves, and differences between strata were tested using the log-rank test. To control for additional variables related to patient survival, Cox proportional hazards modeling was used. These survival probability models included variables representing the cyclin D1 immunohistochemical superpositivity and CCND1 genotype and were controlled for patient age (in decades) and tumor stage (I/II versus III/IV).

Results

Eight hundred and twenty-three eligible cases were invited to participate; of these, 57 refused to participate, 44 did not complete the questionnaire, and 24 did not provide DNA samples for genotyping studies. Therefore, 698 cases are included in the analysis. Of these cases, pathologic tumor stage data were available on 433 individuals, of which 60 (14%) are stage I, 68 (16%) are stage II, 90 (21%) are stage III, and 215 (50%) are stage IV. Similarly, 1,623 subjects were identified as potential controls, and a total of 777 consented to participation, completed the questionnaire, provided a DNA sample, and are thus included in the analysis.

Table 1 describes the characteristics of the study population. As expected, the mean age and distribution of gender were similar between cases and controls. As previously described (20, 21), there was an increasing relative risk of HNSCC with increasing pack-years smoked, with individuals in the highest quartile having an OR of 3.7 [95% confidence interval (95% CI), 2.7-5.1]. The estimated magnitude of cancer risk for tobacco use was similar for oral and pharyngeal cancers but was greatly elevated (OR, 12.6; 95% CI, 6.2-25.5) for laryngeal cancer. There was a nonlinear dose response for HNSCC risk with increasing lifetime average drinks per week. Compared with subjects drinking <2.5 drinks per week on average, those with low average weekly alcohol consumption (2.5 to <6 drinks per week) had a significantly reduced relative risk of HNSCC overall (OR, 0.6; 95% CI, 0.4-0.9). This was observed in all subsites but was statistically significant only among the oral cancers (OR, 0.6; 95% CI, 0.4-0.9). At the same time, subjects whose lifetime average alcohol consumption was >14 drinks per week had a significantly elevated overall relative risk for HNSCC (OR, 2.2; 95% CI, 1.5-3.1), with similar ORs across the subsites. In addition, as previously reported (6), seropositivity for HPV16 was associated with significantly increased HNSCC risk (OR, 4.4; 95% CI, 3.1-6.3), with the greatest OR for tumors of the pharynx (OR, 8.3; 95% CI, 5.1-13.7).

We examined the effect of the variant CCND1 genotype on a phenotypic marker in tumors from patients harboring this genotype, as this variant has previously been associated with
show a lesser association with HPV16 serology, we examined significantly associated with disease in the tumor subsites that not shown). As the homozygous variant genotype was tobacco smoking, alcohol use, or HPV16 seropositivity (data

to control for confounders. This effect was most prominent in oral HNSCC overall (OR, 1.5; 95% CI, 1.1-2.0) in models con-

There was no significant interaction of this genotype with was associated with a significantly increased relative risk for was altered by this polymorphism, we sought to determine if it was associated with HNSCC in our population. The prevalence of the homozygous wild-type (GG) or heterozygous (GA) individuals

As our data strongly indicated that the protein expression of overall survival than those not associated with HPV (6, 25).

The inverse relationships of HPV status (both seropositivity and tumor DNA) with cyclin D staining led us to examine if association between cyclin D1 superpositivity is only among those negative for HPV16 DNA (6, 25). Results of Kaplan-Meier analysis of CCND1 G870A genotype on overall patient survival are presented in Fig. 2. Among cases, those with the CCND1 AA genotype had significantly better overall survival (P < 0.05, log-rank test; Fig. 2A) compared with those with the GC or GA genotypes. Stratifying by tumor stage, this effect seems most strikingly among the higher-stage tumors but is no longer statistically significant (Fig. 2B and C). Stratifying by HPV16 seropositivity, we find that the association of CCND1 genotype with survival is present and strongly significant (P < 0.01, log-rank test; data not shown) only among HPV16 seronegative cases.

Dichotomizing cyclin D1 immunohistochemistry as in Table 2 showed no significant difference in overall patient survival (Fig. 3A). On the other hand, comparing superpositive staining tumors with all others, there was a trend toward poorer overall survival in the superpositive tumors (Fig. 3C). This effect was highly significant among the stage III/IV tumors (P < 0.03, log-rank test; Fig. 3D) but was not apparent in the stage I/II cases (Fig. 3C). Stratified by CCND1 genotype, the association between cyclin D1 superpositivity is only among

Table 1. Variant CCND1 genotype is associated with oral and laryngeal cancers

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All HNSCC</th>
<th>Oral cancer (n = 355), OR (95% CI)</th>
<th>Pharyngeal cancer (n = 202), OR (95% CI)</th>
<th>Laryngeal cancer (n = 139), OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls (n = 777)</td>
<td>Cases (n = 698)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y), mean (SD)</td>
<td>61.0 (11.3)</td>
<td>59.9 (11.8)</td>
<td>1.0 (reference)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td>Male</td>
<td>568 (71.9)</td>
<td>530 (72.6)</td>
<td>1.3 (0.8-2.1)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>222 (28.1)</td>
<td>200 (27.4)</td>
<td>1.3 (0.9-2.0)</td>
</tr>
<tr>
<td>Race, n (%)</td>
<td>Non-White</td>
<td>69 (8.7)</td>
<td>81 (11.1)</td>
<td>2.4 (1.6-3.5)</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>721 (91.3)</td>
<td>649 (88.9)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>Tobacco (lifetime pack-years), n (%)</td>
<td>≤1</td>
<td>302 (38.2)</td>
<td>147 (20.1)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td></td>
<td>&gt;1 to ≤9</td>
<td>94 (11.9)</td>
<td>60 (8.2)</td>
<td>1.4 (0.9-2.1)</td>
</tr>
<tr>
<td></td>
<td>&gt;9 to ≤34</td>
<td>199 (25.2)</td>
<td>166 (22.7)</td>
<td>1.6 (1.2-2.2)</td>
</tr>
<tr>
<td></td>
<td>&gt;34</td>
<td>195 (24.7)</td>
<td>357 (49.0)</td>
<td>3.7 (2.7-5.1)</td>
</tr>
<tr>
<td>Alcohol use (lifetime average drinks weekly), n (%)</td>
<td>&lt;2.5</td>
<td>178 (22.5)</td>
<td>127 (17.4)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td></td>
<td>2.5 to &lt;6</td>
<td>218 (27.6)</td>
<td>98 (13.4)</td>
<td>0.6 (0.4-0.9)</td>
</tr>
<tr>
<td></td>
<td>6 to &lt;14</td>
<td>194 (24.6)</td>
<td>129 (17.7)</td>
<td>0.9 (0.6-1.3)</td>
</tr>
<tr>
<td></td>
<td>≥14</td>
<td>200 (25.3)</td>
<td>375 (51.5)</td>
<td>2.2 (1.5-3.1)</td>
</tr>
<tr>
<td>HPV16 seropositivity, n (%)</td>
<td>No</td>
<td>478 (68.9)</td>
<td>308 (46.0)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>58 (10.8)</td>
<td>145 (21.0)</td>
<td>4.4 (3.1-6.3)</td>
</tr>
<tr>
<td>CCND1 genotype</td>
<td>Wt/Wt + Wt/Var</td>
<td>634 (81.6)</td>
<td>524 (75.1)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td></td>
<td>Var/Var</td>
<td>143 (18.4)</td>
<td>174 (24.9)</td>
<td>1.5 (1.1-2.0)</td>
</tr>
</tbody>
</table>

NOTE: Models are controlled for all variables in table. Two cases did not have ICD9 coding and thus were not included in location-specific models.

*Serum HPV antibody status was available for 989 subjects. Remaining subjects were coded as missing and included in the model.
the CCND1 GG or GA genotype individuals (P < 0.07, log-rank test; data not shown), whereas there was no association between cyclin D1 immunohistochemical superpositivity and survival among the variant AA genotype cases. To examine these two measures simultaneously, as well as to control for confounders of survival, we used a multivariate Cox proportional hazards model of survival (Table 3), controlling for patient age and tumor stage. We have chosen to control for tumor stage in these models, instead of specific treatment regimens, as there is significant colinearity between stage and treatment plans, with low-stage cases being treated with either surgery (majority) or radiation alone, whereas higher-stage (III/IV) disease is treated with both surgery and radiation as well as chemotherapeutic approaches (26). This model suggests a significantly reduced hazards ratio (P < 0.05) for CCND1 AA genotype (hazard ratio, 0.6; 95% CI, 0.3-1.0) compared with AG and GG genotypes and a significantly increased hazard ratio (P < 0.05) for superpositive staining (hazard ratio, 3.6; 95% CI, 1.0-13.0).

Discussion

Our initial work defined an additional phenotypic consequence of the CCND1 polymorphism, showing that cases possessing the homozygous variant genotype had an increased

**Table 2.** Altered cyclin D immunohistochemical staining is associated with variant genotype and inversely associated with tumor HPV positivity in HNSCC cases

<table>
<thead>
<tr>
<th>Tumor characteristic</th>
<th>Cyclin D1 immunohistochemical staining</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative or equivocal (n = 17), n (%)</td>
<td>Positive or superpositive (n = 98), n (%)</td>
</tr>
<tr>
<td>CCND1 genotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt/Wt + Wt/Var</td>
<td>17 (100)</td>
<td>74 (75.5)</td>
</tr>
<tr>
<td>Var/Var</td>
<td>0 (0)</td>
<td>24 (24.5)</td>
</tr>
<tr>
<td>Tumor HPV16 DNA status †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>7 (46.7)</td>
<td>71 (74.7)</td>
</tr>
<tr>
<td>Present</td>
<td>8 (53.3)</td>
<td>24 (25.2)</td>
</tr>
</tbody>
</table>

*Fisher’s exact test.
†Tumor HPV16 status available on 110 tumors.
prevalence of positive or superpositive immunohistochemical staining for the cyclin D1 protein (Table 2). This is consistent with the molecular evidence that the variant A allele codes for an alternate transcript, whose protein product exhibits a greater half-life compared with that encoded by the G allele (15). These findings, taken together, provide an excellent grounding for further study of this variant in a case-control studies of cancer risk.

We also found, in one of the largest studies to date, that the variant AA CCND1 genotype was associated with an increased relative risk of HNSCC. The magnitude of the risk estimate for this variant AA genotype of 1.5 is similar to what has been previously reported in a smaller hospital-based study of HNSCC (16) but lower than that reported for the association between this genotype and oral premalignant lesions (17). At the same time, our results are opposite to those reported by Holley et al. (18) in a German population, who list the G allele as variant and find significantly elevated risk (OR, 3.37; 95% CI, 1.61-9.80) in subjects possessing the GG genotype for oral squamous cell carcinomas (18). Interestingly, Matthias et al. (19) observed no association of this genotype with cancer risk in a population of predominantly laryngeal cancers (19). These studies, however, controlled only for age and gender in their logistic regression analyses, and thus, the differences observed between their results and ours may be due to a large level of residual confounding in their model that did not control for smoking, alcohol consumption, or HPV16.

Our observation that the risk associated with the CCND1 variant allele is greatest in oral and laryngeal, but not pharyngeal, is also of interest, particularly as this is opposite to what we have observed with HPV16 seropositivity. HPV acts as a carcinogen through the expression of the E6/E7 proteins, which inactivate the tumor suppressor proteins p53 and pRB, among other functions (27). Patients possessing the variant cyclin D1 protein may already be predisposed to abrogation of the pRB protein product through increased or longer-lived expression of the cyclin D1 protein, which, through interaction with cyclin-dependent kinase 4/6, leads to the phosphorylation and subsequent degradation of pRB. The inverse relationship between cyclin D1–positive immunohistochemical staining and HPV16 DNA we observed in the primary tumor samples adds additional strong evidence for this hypothesis.

We also observed that cases possessing the AA genotype had significantly better overall survival compared with those having the GA or GG genotypes (Fig. 2A), and this effect was evident among higher-stage disease. This result is consistent with the report of Matthias et al. (19) who observed poorer survival with the GG genotype. We have previously shown that HPV16 seropositive cases have significantly better survival rates, and consistent with other work, these cases are more often pharyngeal (6). Interestingly, if our population of cases is stratified by HPV16 seropositivity, we find that the association of CCND1 genotype with survival is present and strongly significant only among HPV16 seronegative cases. This suggests that possibly the same survival advantage, be it less aggressive...
disease or better treatment response, associated with HPV may also be present in the individuals with variant genotype.

At the same time, cyclin D1 protein immunohistochemical superpositivity was related to poorer patient survival, achieving statistical significance in the high-stage disease. Although this seems at odds with the observation that variant CCND1 genotype is significantly associated with positive staining, if the survival analysis is stratified by CCND1 genotype, the association between cyclin D1 superpositivity is only among the CCND1 GG or GA genotype individuals (P < 0.07, log-rank test; data not shown). There is no appreciable relationship among the variant AA genotype cases. These results suggest that the superpositively staining may be resulting from different molecular events than those related to more moderate immunohistochemical positivity. This is borne out in the multivariate proportional hazards model, which shows independent effects of CCND1 genotype and cyclin D1 immunohistochemical superpositivity on survival (Table 3).

We have observed a genotype-phenotype correlation suggesting that the AA genotype of CCND1 G870A polymorphism is associated with positive immunohistochemical staining for the cyclin D1 protein in HNSCC tumors. This motivated our examination of the association between CCND1 genotype and HNSCC, where we have observed a 50% increased relative risk of HNSCC in individuals harboring the CCND1 AA genotype independent of the risks associated with tobacco smoking.
alcohol use, and HPV16 infection. Further, cases with the homozygous variant genotype seem to have better overall survival, suggesting that these individuals may be similar to those with HPV-related HNSCC, as they also experience enhanced survival from this disease. Finally, we have noted that superpositive immunohistochemical staining for cyclin D1 protein may be a potential prognostic marker of poorer survival in HNSCC, but additional larger studies must be undertaken to confirm these findings.

Acknowledgments

We thank the collaborating clinicians and research staff involved in this study.

References

A Genotype-Phenotype Examination of Cyclin D1 on Risk and Outcome of Squamous Cell Carcinoma of the Head and Neck


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/8/2371

Cited articles
This article cites 26 articles, 9 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/8/2371.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/14/8/2371.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.