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Cyclin D1 Polymorphism and Expression in Patients with Squamous Cell Carcinoma of the Head and Neck

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We have previously reported that the cyclin D1 (*CCND1*) GG^{870} genotype was associated with poorly differentiated tumors and reduced disease-free interval in patients with squamous cell carcinoma of the head and neck (SCCHN). We have now examined the association of this and a second *CCND1* polymorphism with gene expression and outcome in SCCHN patients. Analysis of a *CCND1* G/C^{1722} polymorphism revealed that *CCND1* CC^{1722} genotype was associated with poorly differentiated tumors [*P* = 0.005; odds ratio (OR), 5.7; 95% CI, 1.7 to 19.2), and reduced disease-free interval (*P* = 0.003; Hazard Ratio (HR), 7.3; 95% CI, 1.1 to 27.2.) independently from the influence of *CCND1* GG^{870} genotype. Patients whose tumors were negative for cyclin D1 were associated with reduced disease-free interval (*P* = 0.028; HR, 4.1; 95% CI, 1.4 to 14.2). Although G/C^{1722} genotypes were not associated with expression, we found a significant trend between reduced expression of cyclin D1 in patients with the *CCND1* GG^{870} genotype (*P* = 0.04). Splicing of *CCND1* mRNA in head and neck tissues was modulated by *CCND1* A/G^{870} alleles, thus *CCND1 transcript a* was spliced equally from *CCND1* A^{870} and G^{870} alleles, whereas *CCND1 transcript b* was spliced mainly from the *CCND1* A^{870} allele. Our analysis has also identified differences in cyclin D1 genotype and protein expression and the pathogenesis of SCCHN in males and females. Thus, *CCND1* CC^{1722} genotype was more common in female patients (*P* = 0.019; OR, 3.3; 95% CI, 1.3 to 10) and cyclin D1 expression was more frequent (chi-square$_1$, 3.96; *P* = 0.046) and at higher levels (*P* = 0.004) in tumors from female patients. In summary, our data show that the two *CCND1* polymorphic sites are independently associated with tumor biology and clinical outcome. *CCND1* A/G^{870} alleles affect gene expression in head and neck tissues. We also provide preliminary evidence that the molecular genetics of SCCHN development may be influenced by patient gender. *(Am J Pathol 2001, 159:1917–1924)*

Squamous cell carcinoma of the head and neck (SCCHN) comprise ~5% of newly diagnosed malignancies in Northern Europe and the United States. Annually, more than 500,000 new cases are registered worldwide and the incidence of the disease is increasing.[1] Survival rates for the disease are poor, clinical outcome can vary among patients with tumors from the same site, with comparable tumor stage, nodal status, and histological grade.[2–4] Chronic consumption of tobacco and alcohol are recognized risk factors although it is unclear which traits determine tumor behavior and therefore prognosis. Studies have demonstrated elevated levels of cell proliferation in a high proportion of SCCHN tumors, and proliferation rates have been related to patient survival, and used in patient treatment strategies.[5–7] Thus, genes that encode regulators of cell proliferation may prove useful in establishing patient prognosis or as targets in therapy regimens.

The cyclin D1 gene (*CCND1*) encodes cyclin D1 protein, which is expressed in response to mitogenic signals promoting transition through the restriction point in the G_1 phase of the cell cycle.[8] Increased expression of cyclin D1 has been associated with increased cell proliferation.[9,10] *CCND1* amplification leading to deregulated *CCND1* expression is common in tumors from patients with SCCHN.[11,12] Cyclin D1 protein overexpression has been shown to correlate with reduced 5-year and overall survival in SCCHN patients.[13] Other studies have shown that cyclin D1 protein overexpression is associated with

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poor prognosis in primary hypopharyngeal, laryngeal, esophageal, and oral squamous cell carcinomas.[14–16] Furthermore cyclin D1 antisense experiments have demonstrated that the gene may be a potential target for therapeutic intervention in SCCHN.[17]

CCND1 is polymorphic with a common A/G substitution at nucleotide 870 in the conserved splice donor region of exon 4 of the gene.[18] *CCND1* alleles have been shown to be associated with splicing of *CCND1* mRNA in both normal and tumorous lung tissue. In heterozygotes, *transcript a* is spliced equally from *CCND1* G^{870} and A^{870} alleles, whereas *transcript b* is spliced mainly from the *CCND1* A^{870} allele.[18] In non-small cell lung cancer patients, *CCND1* AA^{870} genotype is associated with poor prognosis.[18] In contrast we have recently demonstrated in SCCHN that the *CCND1* GG^{870} genotype was associated with poorly differentiated tumors and independently from tumor differentiation, with reduced patient disease-free interval.[19,20] A second common G/C polymorphism at nucleotide 1722 within *CCND1* 3′UTR has also been described (G/C^{1722} sequence information is available at the NCBI SNP database at *http://www.ncbi.nlm.nih.gov/SNP*), although the influence of this polymorphism on tumorigenesis has not been examined.[21]

In this study we have further investigated the role of *CCND1* allelism in SCCHN. We have examined the relationship of the *CCND1* G/C^{1722} polymorphism with A/G^{870} alleles and clinical outcome. In addition we have further studied the influence of *CCND1* alleles on mRNA splicing and protein expression in head and neck tissue.

Materials and Methods

Patients

SCCHN patients ($n = 294$) were studied from our original cohort of 384 patients previously described.[19,20] These patients were selected because of the availability of DNA and do not represent a clinical subgroup. The clinical pathological characteristics are similar to those of the total cohort.[19] Briefly, the case group comprised German Caucasians suffering a single histologically confirmed oral cavity pharyngeal or laryngeal squamous cell carcinoma. Patients were recruited at first presentation or during follow-up between 1994 and 1996. Malignancies were staged using the TMN classification system.[22] All tumors were squamous cell carcinoma and were histologically graded as: well (G_{0-1}), moderately (G_2), and poorly (G_3) differentiated. Margins of the resected specimen were examined by a histopathologist and judged for tumor-free margins using the international R0-R2 system; R0, microscopically proven free tumor margins; R1, microscopic infiltration and macroscopically free margin; R2, macroscopic tumor infiltration of the margin. Where details were available we examined the association of cyclin D1 genotype and protein expression with factors known to influence clinical outcome: tumor site ($n = 268$), tumor size (T1 to T4) ($n = 258$), histological differentiation ($G_{0/1}$ to G_3) ($n = 168$) and the presence of nodes at time of surgery ($n = 166$). We also examined the association of genotypes and protein expression with tumor recurrence (defined as disease-free interval) in 151 patients. The study to identify associations with disease-free interval was performed only in patients in which a R0 resection could be achieved during initial treatment. Patients suffering a tumor re-growth during the first 6 months after initial treatment and those suffering extra-capsular tumor spread in any of the resected lymph nodes were excluded to avoid misjudged recurrences because of residual tumor growth. Lymph node involvement was determined before surgery using ultrasound, computed tomography, and nuclear magnetic resonance imaging and later histologically proven in the neck dissection specimen.

DNA and RNA Extraction

Peripheral blood samples were collected in ethylenediaminetetraacetic acid. Tumor and histologically normal head and neck tissues (salivary gland or muscle) were collected at time of surgery and snap-frozen in liquid nitrogen and stored at −70°C before use. DNA was extracted from frozen tissue and blood using a phenol-chloroform method.[23] mRNA was isolated from frozen normal and tumor tissues using the MicrofastTrack RNA isolation kit (Invitrogen, Groningen, The Netherlands).

Restriction Fragment Length Polymorphism-Polymerase Chain Reaction (PCR) Genotyping

CCND1 G/C^{1722} genotypes were identified in DNA isolated from peripheral blood using a restriction fragment length polymorphism-PCR based assay.[24] Briefly reactions were performed in 25 μl containing 1× *Taq* polymerase buffer (Promega, Southampton, UK), 100 μmol/L dNTPs, 0.25 μg of each primer, 2% dimethyl sulfoxide, 0.25 U of *Taq* polymerase (Promega, UK) and 0.1 μg of DNA. Reactions were performed on an automated thermal cycler with an initial denaturation of 94°C (2 minutes) and cycled 34 times with annealing temperature of 57°C (1 minute), an extension at 72°C (1 minute), and denaturation of 94°C (1 minute). PCR products were digested with *Hae*III following the manufacturer's guidelines (New England Biolabs, Hitchin, Hertfordshire, UK). Alleles were resolved on 3% agarose gels stained with ethidium bromide.

Reverse Transcriptase-PCR Analysis of CCND1 mRNA

mRNA extracted from snap-frozen tissues was reverse-transcribed to cDNA using a superscript preamplification system (Gibco-BRL Life Technologies, Paisley, UK). Analysis of *CCND1* transcripts *a* and *b* was performed using a nested PCR strategy.[18] Reactions containing 2 μl of cDNA were performed in 100-μl volumes containing 1× PCR buffer (Promega), 100 μmol/L dNTPs, 0.5 μg each primer, 2 U of *Taq* polymerase (Promega). Primary reactions were cycled 32 times, following which 1 μl of

Table 1. Distribution of *CCND1* G/C^{1722} Genotypes and Tumor Protein Expression in SSCHN Patients

	CCND1 G/C^{1722} genotype			Cyclin D1 protein expression	
	GG (%)	GC (%)	CC (%)	Positive* (%)	Negative (%)
Total cases	117 (39.8)	141 (48.0)	36 (12.2)	112 (83.0)	23 (17.0)
Male	106 (41.7)	121 (47.6)	27 (10.6)	87 (79.8)	22 (20.2)
Female†	11 (27.5)	20 (50.0)	9 (22.5)	25 (96.1)	1 (3.9)
Tumor site					
Laryngeal SCC	81 (41.5)	90 (46.2)	24 (12.3)	56 (82.4)	12 (17.6)
Pharyngeal SCC	16 (32.7)	26 (53.1)	7 (14.3)	27 (81.8)	6 (18.2)
Oral Cavity SCC	9 (37.5)	12 (50.0)	3 (12.5)	13 (81.2)	3 (18.8)
Multiple sites	11 (42.3)	13 (50.0)	2 (7.7)	16 (88.9)	2 (11.1)
T-factor					
T1	36 (38.7)	51 (54.8)	6 (6.5)	22 (73.3)	8 (26.7)
T2	27 (42.2)	29 (45.3)	8 (12.5)	25 (92.6)	2 (7.4)
T3	21 (42.0)	21 (42.0)	8 (16.0)	14 (100)	0 (0.0)
T4	19 (37.3)	25 (49.0)	7 (13.7)	31 (75.6)	10 (24.4)
Histological differentiation‡					
Well	5 (55.6)	4 (44.4)	0 (0.00)	5 (71.4)	2 (28.6)
Moderate	47 (40.2)	58 (49.6)	12 (10.3)	45 (76.3)	14 (23.7)
Poor	11 (26.2)	21 (50.0)	10 (23.8)	24 (80.0)	6 (20.0)
Lymph nodes					
Negative	38 (40.0)	49 (51.6)	8 (8.4)	43 (79.6)	11 (20.4)
Positive	23 (32.4)	34 (47.9)	14 (19.7)	30 (76.9)	9 (23.1)

*Positive protein expression was defined as >10% tumor cells expressing cyclin D1 and was more frequent in tumors from female patients (chi-square$^2_1$ = 3.96; P = 0.046).

†Using logistic regression analysis corrected for imbalances in age, with *CCND1* GG^{1722} as a reference, the *CCND1* CC^{1722} genotype was more common in female patients P = 0.025; OR, 3.3; 95% CI, 1.2 to 9.51.

‡Using logistic regression correcting for age and gender in the model *CCND1* CC^{1722} was associated with poor tumor differentiation (G_0/1, G_2 *versus* G_3), P = 0.005; OR, 5.7; 95% CI, 1.7 to 19.2.

PCR product was nested for 22 cycles. PCR conditions were initial denaturation temperature 94°C (2 minutes), annealing 55°C (1 minute), elongation 74°C (1 minute), and denaturation 94°C (1 minute). PCR products were digested with the restriction enzyme *ScrF*I (New England Biolabs) and alleles visualized on 8% acrylamide gels and silver stained.

Immunohistochemistry

Paraffin-embedded tumor material was available in 135 cases from the cohort of 294 patients. Immunohistochemistry was performed using a Shandon Sequenza (Shandon Scientific Limited, Cheshire, UK) as described.[25] A mouse monoclonal cyclin D1 antibody (DCS-6; Novocastra, Newcastle-upon-Tyne, UK) was used. A cyclin D1-positive breast tumor provided positive control material. Negative controls had no primary antibody applied and included a section of normal head and neck tissue. Slides were graded as negative (0 to <10% cells stained), low (>10 to 50% cells stained), moderate (>50 to 75% cells stained), and strong (>75% cells stained).

Statistics

Statistical analysis was performed using Stata, version 5 (Stata Corporation, College Station, TX). Pearson chi-square tests were used to identify linkage between *CCND1* genotypes and associations of genotypes with gender. Association of genotypes and protein expression with clinicopathological parameters were analyzed and corrected for imbalances in age and gender using logistic regression. Associations between cyclin D1 protein and genotype and cyclin D1 protein expression with gender were analyzed using the Armitage trend test. Cox's proportional hazard regression model was used in the analysis of the effects of cyclin D1 staining and genotypes on disease-free interval. Kaplan-Meier curves were generated for graphical representation of associations with disease-free interval. A probability level of 5% was considered statistically significant.

Results

The frequencies of *CCND1* G/C^{1722} genotypes in 294 patients with SCCHN are shown (Table 1). Allele frequencies conformed to Hardy Weinberg equilibrium. Furthermore significant linkage disequilibrium was demonstrated between G/C^{1722} and A/G^{870} alleles, thus 32 of 34 (94%) individuals with the *CCND1* CC^{1722} genotype were also GG^{870} (Table 2). The distribution of *CCND1* G/C^{1722} genotypes was significantly different between female and male patients with the *CCND1* CC^{1722} genotype being more common in female patients (Table 1). This associ-

Table 2. Association of *CCND1* A/G^{870} and G/C^{1722} Genotypes in SCCHN Patients

	CCND1 A/G^{870} genotypes		
CCND1 G/C^{1722} genotypes	AA (%)	AG (%)	GG (%)
GG (%)	54 (47.4)	49 (43.0)	11 (9.6)
G/C (%)	11 (8.1)	87 (64.0)	38 (27.9)
CC (%)	0 (0.0)	2 (5.9)	32 (94.1)

Significant linkage disequilibrium was demonstrated between *CCND1* G/C^{1722} and A/G^{870} alleles (P < 0.001; chi-square$_4$ = 136.3).

Table 3. Association of Cyclin D1 Expression in Tumors with *CCND1* A/G^{870} Genotypes and Patient Gender in SCCHN

	Patient gender		*CCND1* genotype	
Cyclin D1 protein*	Female†	Male	*CCND1* AG^{870}/AA^{870}	*CCND1* GG^{870}‡
Negative	1 (4.3)	22 (95.7)	14 (63.6)	8 (36.4)
Low	5 (14.3)	30 (85.7)	22 (66.7)	11 (33.3)
Moderate	12 (21.4)	44 (78.6)	42 (79.2)	11 (20.8)
High	8 (38.1)	13 (61.9)	17 (85.0)	3 (15.0)

Figures in parenthesis are percent.
*Levels of cyclin D1 expression are defined as the proportion of tumor cells expressing protein and are, negative (<10%), low (10 to 50%) moderate (50 to 75%) and high (>75%).
†Female patients are associated with high levels of cyclin D1 expression (Armitage test for trend $P = 0.004$).
‡*CCND1* GG^{870} genotype is associated with low expression of cyclin D1 protein (Armitage test for trend, $P = 0.049$).

ation remained significant after correction for smoking and alcohol consumption ($P = 0.036$; OR, 3.7). *CCND1* A/G^{870} genotypes were not associated with patient gender.[19,20]

We next examined the data for associations between the *CCND1* G/C^{1722} genotypes and factors that influence outcome in the total group. There was no association between patient genotype and age at tumor presentation (data not shown) and no association was found between *CCND1* genotypes and tumor stage and tumor site (Table 1). However, we found using logistic regression with correction for age and gender, *CCND1* CC^{1722} genotypes were associated with poorly compared to well and moderately differentiated tumors (Table 1). As before, we found that *CCND1* GG^{870} genotype was associated with poorly differentiated tumors[19] ($P = 0.016$; OR, 2.2; 95% CI, 1.15 to 4.21), however on including both *CCND1* genotypes in a model with age and gender we found *CCND1* CC^{1722} remained significantly associated with tumor differentiation ($P = 0.036$; OR, 4.91; 95% CI, 1.1 to 21.8), whereas *CCND1* GG^{870} was no longer significantly associated ($P = 0.76$; OR, 1.17; 95% CI, 0.43 to 3.23). *CCND1* CC^{1722} genotype was more common in patients with tumor nodes, although this did not achieve statistical significance ($P = 0.075$; OR, 2.8; 95% CI, 0.9 to 8.9). We further examined the association of *CCND1* G/C^{1722} genotypes with differentiation and tumor nodes in the cases with laryngeal tumors. *CCND1* CC^{1722} genotypes were associated with poorly differentiated tumors ($P = 0.006$; OR, 9.2; 95% CI, 1.9 to 44.9) and the presence of tumor nodes in laryngeal tumors ($P = 0.046$; OR, 4.3; 95% CI, 1.0 to 18.1).

Cyclin D1 expression was detected in 112 of 135 (83%) of tumors examined (Table 1). The proportion of tumor cells expressing cyclin D1 was variable between different tumors. Thus expression was low in 35 of 135 (25.9%) cases, moderate in 56 of 135 (41.4%) cases, and high in 21 of 135 (15.5%) cases. Cyclin D1 protein expression was not associated with patient age at presentation neither did we find an association with tumor stage, differentiation, or the presence of tumor nodes (Table 1). Analysis of cyclin D1 expression with patient gender revealed that tumor expression of cyclin D1 was high in both sexes (Table 1). However, significantly more tumors from females expressed cyclin D1 compared with those from males (Table 1). Furthermore, a significant trend was observed between an increase in the proportion of cells expressing cyclin D1 in the tumors from female patients, compared with those from males (Table 3).

We next examined *CCND1* genotype and protein expression data for associations with patient disease-free interval. The Kaplan-Meier plot demonstrated that *CCND1* CC^{1722} genotype was associated with reduced time to tumor recurrence (Figure 1A). Using Cox's proportional hazards model to correct for imbalances in patient age and gender, with *CCND1* GG^{1722} genotype as reference, *CCND1* CC^{1722} was significantly associated with an increased proportion of patients having tumor recurrence after 2 years (Table 4). On including tumor differentiation in the model this association was reduced ($P = 0.042$; HR, 3.76; 95% CI, 1.05 to 13.47). As

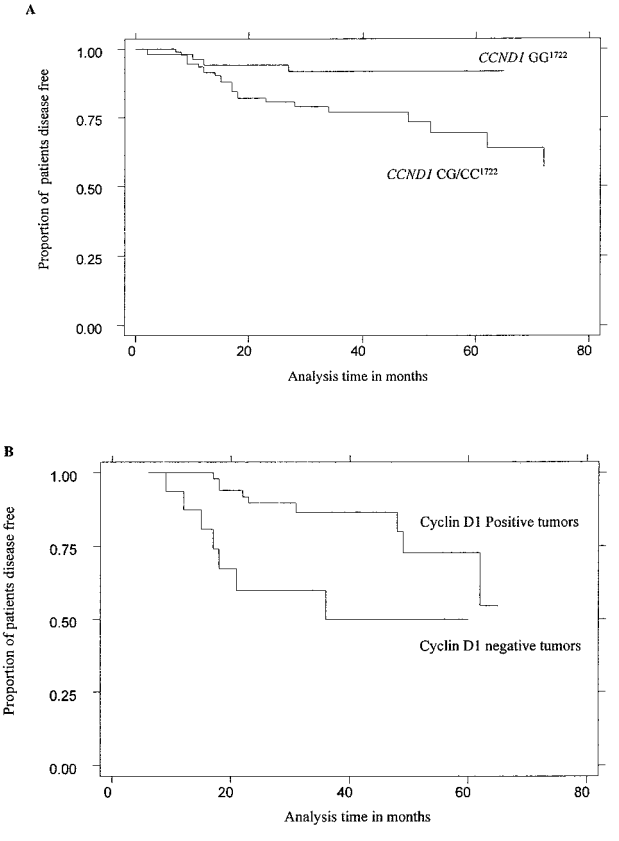

Figure 1. Kaplan-Meier survival plots showing the association between *CCND1* G/C^{1722} genotypes and disease-free interval (**A**) and the association between cyclin-D1 protein and disease-free interval (**B**) in SCCHN patients.

Table 4. Association of *CCND1* Genotypes, Tumor Cyclin D1 Protein Expression, and Prognostic Indicators with Tumor Recurrence in SCCHN

Prognostic indicator	*P*	HR	95% CI	Proportion of patients with recurrence
CCND1 G/C^{1722}*				
GG	ref			5.7
CG	0.362	1.6	0.6–4.8	17.6
CC	0.003	7.3	11.0–27.2	26.9
CCND1 A/G^{870}*				
AA	ref	—	—	5.0
AG	0.456	1.5	0.5–4.2	10.2
GG	0.010	3.9	1.4–11.0	28.0
Cyclin D1 protein*				
Positive	ref	—	—	10.3
Negative	0.028	4.1	1.4–14.2	40.2
Differentiation†				
$G_{1/2}$	ref			9.6
G_3	<0.001	3.1	1.7–5.7	28.2
Stage†				
T1, 1.1,2	ref	—	—	10.3
T3,4	0.134	1.6	0.9–3.1	18.0

**P* values were obtained using Cox's regression corrected for age and gender tumor site and T-stage.
†*P* values were obtained using Cox's regression corrected for age and gender.

previously reported *CCND1* GG^{870} genotype was associated with reduced disease-free interval[19] (Table 4). Including both genotypes in the model, although not significant *CCND1* GG^{870} ($P = 0.12$; HR, 2.0; 95% CI, 0.83 to 4.85) and *CCND1* CC^{1722} ($P = 0.102$; HR, 3.2; 95% CI, 0.8 to 12.9) were both associated with an increase in the proportion of patients suffering a tumor recurrence after 2 years. Patients whose tumors did not express cyclin D1 were associated with reduced disease-free interval (Figure 1B). Accordingly, the tumor recurrence after 2 years was significantly higher in patients in this group (Table 4). Furthermore absence of tumor cyclin D1 protein expression seemed to be associated with disease-free interval in patients with laryngeal tumors ($P = 0.047$; HR, 3.7; 95% CI, 1.01 to 5.89).

We further examined the influence of *CCND1* alleles on *CCND1* expression. Cyclin D1 protein was not significantly associated with *CCND1* G/C^{1722} genotypes (data not shown). However, a higher proportion of tumors displayed low expression of cyclin D1 from patients with the *CCND1* GG^{870} genotype compared with tumors from patients with the *CCND1* AA^{870} and A/G^{870} genotypes (Table 3). The influence of *CCND1* A/G^{870} alleles on *CCND1* mRNA splicing was examined. We examined the expression of *CCND1* mRNA from 13 matched normal and tumor tissue pairs of which 9 were *CCND1* AG ^{870}, 2 were AA^{870}, and 2 were GG^{870} genotypes. Expression of both *CCND1* transcripts was detected in tissues from patients of each genotype (Figure 2, A and B). Restriction fragment length polymorphism analysis of cDNA from heterozygotes demonstrated that *CCND1* A/G^{870} alleles modulate *CCND1* mRNA splicing in head and neck tissues. Thus in nine of nine normal tissues and five of nine corresponding tumors, *CCND1 transcript a* was spliced equally from the *CCND1* A^{870} and *CCND1* G^{870} alleles, however *CCND1 transcript b* was spliced mainly from *CCND1* A^{870}. A representative example of *CCND1* mRNA splicing from three different individuals with the *CCND1* AG^{870} genotype is shown (Figure 3).

Discussion

We have demonstrated for the first time, that polymorphism in the 3′-UTR region of *CCND1* is associated with tumorigenesis and clinical outcome in SCCHN patients. *CCND1* CC^{1722} was associated with poorly differentiated tumors and reduced disease-free interval. We have previously reported that *CCND1* GG^{870} was associated with

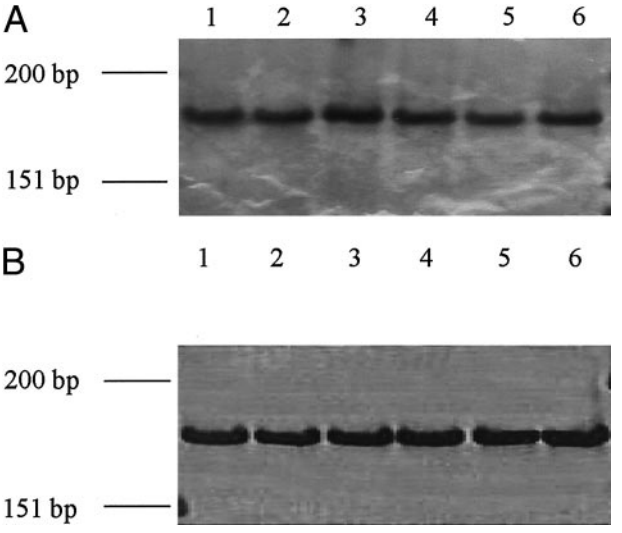

Figure 2. *CCND1* mRNA is alternately spliced in SCCHN patients with different *CCND1* A/G^{870} genotypes. The expression of *CCND1 transcript a* (**A**) and *CCND1 transcript b* (**B**) was detected by PCR on cDNA synthesized from tumors with *CCND1* AA^{870} (**lanes 1** and **2**) *CCND1* AG^{870} (**lanes 3** and **4**) and *CCND1* GG^{870} (**lanes 5** and **6**) genotypes. PCR products were run on polyacrylamide gels and silver stained. ϕX174 *Hin*fI digest was used as a size marker.

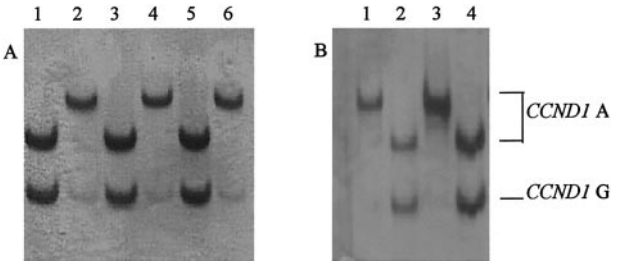

Figure 3. *CCND1* alleles modulate transcript splicing in head and neck tissues. Restriction fragment length polymorphism-PCR analysis was performed on cDNA synthesized from tumor tissues from three patients with the heterozygote *CCND1* AG^{870} genotype (patient 1, **lanes 1** and **2**; patient 2, **lanes 3** and **4**; and patient 3, **lanes 5** and **6**) (**A**), and normal tissues from two of these patients (patient 1, **lanes 1** and **2**; patient 2, **lanes 3** and **4**) (**B**). *CCND1* alleles were resolved through digestion of PCR products with *ScrF*I and separated on polyacrylamide gels and silver stained. *CCND1* alleles are marked with **arrows**. *ScrF*I digests of *CCND1 transcript a*-specific PCR product show *transcript a* is spliced equally from *CCND1* allele A^{870} and G^{870} alleles in both tumor (**A: lanes 1**, **3**, and **5**) and normal (**B: lanes 2** and **4**) tissues; whereas digests of *CCND1 transcript b*-specific PCR products show that *transcript b* is spliced mainly from *CCND1* A^{870} allele in both tumor (**A: lanes 2**, **4**, and **6**) and normal (**B: lanes 1** and **3**) tissues.

poor tumor differentiation and reduced disease-free interval independent from tumor differentiation.[19] However, our preliminary data suggest that although *CCND1* G^{870} and C^{1722} alleles are in linkage disequilibrium, their influence on tumor pathology and patient outcome seems to be separate. Thus *CCND1* GG^{870} is an independent marker for tumor recurrence in SCCHN, whereas *CCND1* CC^{1722} is associated with tumor recurrence by a mechanism associated with tumor biology and mainly through differentiation. Similar associations with tumor differentiation, tumor nodes, and clinical outcome were found with *CCND1* CC^{1722} in laryngeal tumors. However case numbers were not large enough to test for significance in pharyngeal and oral tumors although similar trends were observed.

We have also examined the expression of cyclin D1 protein in these tumors. Cyclin D1 expression was frequently deregulated, with the proportion of cells expressing the protein varying between different tumors. Our data are consistent with findings in other studies.[26,27] However, surprisingly, in our cohort of patients, absence of expression of cyclin D1 correlated with reduced disease-free interval. In contrast previous work has associated up-regulated expression of cyclin D1 with poor outcome in SCCHN.[13–16] Although it is assumed, that deregulated expression leads to poor outcome directly through increased cellular proliferation of tumor cells, in a recent study cyclin D1 expression in SCCHN tumors has been associated with an increase in apoptosis and cyclin D1-negative tumors were more proliferatively active.[27] Cyclin D1 expression has been associated with good prognosis in non-small cell lung cancer and bladder cancer[24,28] and both good and bad prognosis in studies on breast and cancer.[29–32] It is possible that these contradictory results in part reflect the many different mechanisms through which deregulated expression of cyclin D1 can occur in cancer.[11,12,24,29,33] It would be interesting in the future, to examine the association between *CCND1* alleles with the mechanism of protein overexpression such as *CCND1* amplification in addition to biological markers of cellular proliferation and to relate these findings to clinical parameters including tumor differentiation and patient disease-free interval. Further understanding of these mechanisms and their effect on tumor biology in specific tissues may increase understanding of cyclin D1 as a prognostic marker.

To explore mechanisms underlying the link between *CCND1* genotypes and clinical outcome in SCCHN, we investigated the data for associations with gene expression. Firstly, we found a significant trend between a reduction in the proportion of cyclin D1-expressing cells within the tumors of patients with *CCND1* GG^{870}. Furthermore, *CCND1* GG^{870} was associated with reduced disease-free interval in our patients thus providing a link between *CCND1* A/G^{870} alleles with cyclin D1 expression and clinical outcome in SCCHN. Secondly, we found that *CCND1* A/G^{870} alleles modulate the splicing of *CCND1* mRNA in head and neck tissues. *CCND1 transcripts a* and *b* were detected from each *CCND1* A/G^{870} allele. However, *transcript b* was spliced mainly from the *CCND1* A^{870}, whereas *transcript a* was spliced equally from *CCND1* G^{870} and *CCND1* A^{870} alleles. It would be expected therefore, that *CCND1* AA^{870} homozygotes express more *transcript b* than *CCND1* GG^{870} homozygotes. Although we did not measure transcript levels, it is possible that physiological differences in the ratio of *CCND1 transcripts a* to *b* because of interindividual genotypic variation may influence the development of SCCHN. Interestingly, we found that in four of nine of the tumors allele-specific splicing of the transcripts was deregulated (S. L. Holley and P. R. Hoban, unpublished data). It is not clear from this preliminary data whether this is a specific mechanism for tumor development or indicates a general loss of splicing fidelity in these tumors.

CCND1 transcript a is identical to the reported cyclin D1 cDNA,[34] however, *transcript b* fails to splice at the exon 4/intron 4 boundary, terminates downstream of exon 4 and does not contain exon 5.[18] The difference between the proteins predicted by the nucleotide sequences of the two transcripts is in the carboxy terminal PEST rich region (destruction box) encoded by exon 5, which facilitates turnover of the START cyclins.[35] The terminal region of *transcript b* has no PEST-rich sequence. Functional differences in the expression of the alternate *CCND1* transcripts have also been demonstrated.[36] Further, the monoclonal antibody, DCS-6, used in our immunohistochemical analysis, cross-reacts with recombinant cyclin D1 proteins from both *CCND1 transcript a* and *b* and protein from *transcript b* has been detected with this antibody in lymphoma cells and solid tissues.[36–38] Therefore it is not clear in our immunohistochemical analysis which specific protein is being detected in the SCCHN tumors. We did not find any associations between *CCND1* G/C^{1722} alleles and protein expression. Because of its location in exon 5, we assume that *CCND1* G/C^{1722} is a marker reflecting the activity of *CCND1 transcript a.* Alternatively *CCND1* G/C^{1722} alleles may be in linkage with other alleles that influence cyclin D1 function or that of a gene close by.

Our study has highlighted potential differences in the genetics and biology of tumors from SCCHN patients of

different gender. The distribution of G/C^{1722} genotypes was different between male and female patients. We also found that significantly more tumors from female patients and a higher proportion of cells in those tumors expressed cyclin D1, than those from male patients. Our cohort was too small to significantly examine the data for differences in outcome based on *CCND1* and gender. However it was noted that tumor site significantly differed with patient gender. Thus oral (21% female *versus* 7% male) and pharyngeal (38% female *versus* 21% male) tumors were more common in female patients whereas laryngeal tumors were more common in male patients (40% females *versus* 71% males). SCCHN is documented as a predominantly elderly male disease associated with heavy smoking and high alcohol consumption.[1–3] In our cohort, female patients were associated with less alcohol (30% females compared with 73% of males) and tobacco (62% females compared with 88% males) consumption. This data would indicate that SCCHN etiology in males and females may be different. The role of cyclin D1 in tumor development in patients of differing gender is unclear and the gene may merely be a marker for these differences. However, *CCND1* amplification and protein expression has been related to tobacco exposure in SCCHN.[39] In breast cancer patients cyclin D1 expression and estrogen receptor status are significantly correlated and associated with clinical outcome.[40] Furthermore, previous functional studies have suggested that cyclin D1 interacts in a ligand-specific manner with the estrogen receptor in breast cells and the androgen receptor in prostate cells.[41,42]

In conclusion, accumulating data demonstrate that *CCND1* is important in the development of SCCHN and that the gene has potential both as a prognostic marker, and as a target in the treatment of the disease. Our data suggest that *CCND1* A/G^{870} alleles influence expression of *CCND1*, and that specific antibodies to *transcript b* are required to validate expression studies and to identify the specific cyclin D1 protein in SCCHN tumors. We realize, in some cases, numbers were small, and that our findings require independent confirmation in a separate and larger cohort, however, for the first time, we demonstrate the potential of *CCND1* G/C^{1722} alleles as an independent prognostic marker in this disease. Our data also highlights differences in the molecular genetics of SCCHN tumors associated with patient gender. Collectively these findings have implications in rationalizing the protein as a target for treatment of patients with SCCHN.

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