# Cyclin D1, Glutathione S-Transferase, and Cytochrome P450 Genotypes and Outcome in Patients with Upper Aerodigestive Tract Cancers: Assessment of the Importance of Individual Genes Using Multivariate Analysis[1]

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## Abstract

***GST*, *CYP*, and *CCND1* genotypes have been associated with outcome in several cancers. Accordingly, we have examined, in patients with one squamous cell carcinoma (SCC) of the head and neck, associations between *GSTM1*, *GSTT1*, *GSTM3*, *GSTP1*, *CYP2D6*, *CYP1A1*, *CYP2E1*, and *CCND1* genotypes and the outcome parameters, tumor extension, histological grade, and presence of nodes. We used logistic regression to study, first, each gene individually and, second, in a step-wise model that included all of the genes. Different genes were associated with each outcome parameter. Thus, *GSTT1 null* was associated with T3/T4 lesions in the oral cavity/pharyngeal ($P = 0.029$), but not laryngeal, SCC cases. *GSTT1 null* was also associated with histological differentiation (G3) in the oral cavity/pharyngeal, but not laryngeal, SCC cases, although this association only approached significance ($P = 0.069$). *CCND1 GG* was associated with G3 tumors in the oral cavity/pharyngeal ($P = 0.011$), but not laryngeal, SCC cases. The combination of *GSTT1 null/CCND1 GG* was also associated with G3 tumors. *CYP2D6 PM* and *HET* were associated with lymph node involvement in the laryngeal, but not oral/pharynx, SCC cases. Genes that were individually associated with outcome were also associated with the parameter in the step-wise routine. The *GSTT1 null* frequency was greater in 39 patients with second primary tumors than in those with one lesion ($P = 0.014$). The data demonstrate site-dependent associations between *GSTT1 null*, *CCND1 GG*, and *CYP2D6 PM* and tumor extension, differentiation, and nodes.**

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## Introduction

SCCHN[3] comprises about 5% of new cancers in Western countries. Chronic tobacco and alcohol consumption are dose-dependent, causative factors, although not all patients have been exposed and many individuals with excessive consumption do not develop these lesions (1–4). Prognosis is determined by patient (age at diagnosis, gender, medical status) and tumor (size and site of primary) characteristics, as well as treatment (response to surgery). Thus, whereas large lesions with metastatic spread are traditionally considered to have a worse prognosis than small, locally restricted tumors, outcome can vary markedly between patients with tumors from the same site and comparable stage, nodal status, and histological grade (1–4). SCCHN, therefore, offers an opportunity to study the influence of genes on susceptibility and outcome. Selection of candidates is problematical, although previous studies have suggested the relevance of the *GST*, *CYP*, and D-type cyclin supergene families (5–13).

*GST* and *CYP* genes are candidates for susceptibility in SCCHN because they catalyze the detoxication of many relevant electrophiles (14, 15). Their importance is indicated by studies showing a significantly increased frequency of *GSTM1 null* and a nonsignificantly increased frequency of *GSTT1 null* in 186 SCCHN cases, compared with 42 controls (5). We also found a higher frequency of *GSTT1 null* in SCCHN cases than controls, particularly in oral cavity/pharyngeal SCC cases compared with controls or laryngeal SCC cases (although these differences did not quite achieve significance; Ref. 9). Several studies have indicated a role for *GSTM1 null* in SCCHN (5, 6). We found that although the differences were not significant, the frequency of this genotype was rather higher in the oral cavity/pharyngeal SCC (58.2%) and laryngeal SCC (57.0%) cases than in controls (53.4%; Ref. 9). However, Park *et al.* (10) found no difference in the frequency of *GSTM1 null* in oral cavity SCC cases and controls. Other polymorphic *GST* and *CYP* genes have also been associated with SCCHN risk, although in some cases results are discrepant; for example, *GSTM3 AA* with risk for laryngeal SCC, *GSTP1* $Ile^{105}/Ile^{105}$ with reduced risk of oral/pharyngeal SCC and the exon 7 *ile/val CYP1A1* polymorphism, and *CYP2D6 PM* with susceptibility to oral cavity cancer (8, 10, 16, 17).

The proto-oncogene *CCND1* gene product regulates transition through the $G_1$-S checkpoint and is a further candidate for cancer outcome/susceptibility. Indeed, deregulated expression

[3] The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; CYP, cytochrome P450; GST, glutathione S-transferase; CCND1, cyclin D1; OR, odds ratio; CI, confidence interval; PM, poor metabolizer; HET, heterozygote phenotype; EM, extensive metabolizer phenotype.

of *CCND1* and aberrations at this checkpoint are common in malignancy and have been associated with outcome in SCCHN (18–21). Importantly, an intragenic A/G polymorphism in *CCND1* seems to modulate splicing of *CCND1* mRNA. Transcript *a* is identical to the reported cyclin D1 cDNA, whereas transcript *b* fails to splice at the exon 4/intron 4 boundary and terminates downstream of exon 4 (22). The proteins encoded by the transcripts differ in the COOH-terminal PEST rich region (destruction box) encoded by exon 5, which is responsible for rapid turnover of the START cyclins. Studies in the lung show that the *CCND1* genotype influences the ratio of the two transcripts (22). The relevance of this polymorphism is shown by the finding that *CCND1 GG* is an independent prognostic indicator of disease-free interval, but not susceptibility, in SCCHN cases (7).

Recent studies suggest that various GST and CYP genotypes are linked with cancer outcome rather than susceptibility (15, 23). Indeed, both *CYP2D6 PM* and *CCND1 GG* demonstrate site-specific associations with outcome in SCCHN (7, 16). Accordingly, we have assessed the influence of: (*a*) individual *GSTM1*, *GSTM3*, *GSTT1*, *GSTP1*, *CYP2D6*, *CYP1A1*, *CYP2E1* and *CCND1* genotypes; and (*b*) using a step-wise model that included all of the genes to determine, on the basis of *P* and rate ratio, the relative importance of individual genotypes. For each gene, we factorized genotypes to identify those that conferred increased or decreased risk. Because the influence of polymorphisms on susceptibility varies in different sites (7–9), we separately assessed the influence of genotypes in cases with oral cavity/pharyngeal tumors and laryngeal SCC. In the context of outcome parameters, we studied associations between genotypes and tumor extension, histological grade, and the presence of nodes. We also examined the data for links between genotypes and the development of further tumors because cases with more than one SCCHN are known to be at a genetically determined high-risk (4, 24).

## Materials and Methods

**Patients.** We recruited 423 unrelated German Caucasians from the Berlin region, suffering at least one oral cavity/pharyngeal or laryngeal SCC (7–9). They were recruited with appropriate approval and informed consent at first presentation or during 5-year follow-up between 1994 and 1996 and comprised 91% of the cases admitted to the Department of Otorhinolaryngology, Virchow-Klinikum, during this period. Three hundred eighty-four patients suffered a single, histologically confirmed oral cavity/pharyngeal ($n = 125$) or laryngeal ($n = 259$) SCC. The oral cavity/pharyngeal group comprised 87 patients with tumors of the oropharynx and hypopharynx and 38 cases with oral cavity SCC. In the laryngeal group, patients had supraglottic, glottic, or subglottic tumors. These patients have been described in case-control studies designed to identify associations between *GST* and *CYP* genotypes and susceptibility to oral cavity/pharyngeal and laryngeal SCCHN (8, 9). *GST* and *CYP* genotype frequencies, together with alcohol and tobacco consumption, are described (8, 9). An additional 39 cases with more than one primary SCCHN were also studied. They comprised patients with at least two malignant SCCs separated by a 2-cm histologically normal mucosa or a 2-cm distance from the resection margin (24). Two of 39 patients developed synchronous (within 6 months of the first diagnosis) tumors, and 37 cases developed metachronous (more than 6 months after the first diagnosis) tumors. All cases and controls were interviewed by a surgeon (C. M. or V. J.) familiar with the study aims to obtain information on alcohol and tobacco consumption. Cigarette smoking was graded: never smokers, 1–10 cigarettes/day, 11–20 cigarettes/day, and more than 20 cigarettes/day. Twenty-seven of 423 patients stopped smoking before diagnosis of SCCHN. All cases classified as current or ex-smokers were found to have smoked for at least 25 years. Alcohol consumption was also graded: never, up to 50 g/day, 50–100 g/day, and >100 g/day. Patients suffering multiple primary tumors in the upper aerodigestive tract did not differ from the single tumor group in their smoking and alcohol consumption or in their age and gender distribution.

**Outcome Parameters.** The initial tumor location and extension was recorded using the tumor, node, and metastasis system (Union International Contre Cancer) at diagnosis in all 384 cases with one SCCHN: T1 tumors ≤2 cm in greatest dimension, T2 tumors >2 cm and <4 cm, T3 tumors >4 cm in the greatest dimension, and T4 tumors demonstrated invasion. Further comparisons of genotypes and outcome were made in the 253 cases who were treated with surgery with curative intent. Histological differentiation of the tumor, lymph node involvement of the neck, and confirmation of the residual-free resection were determined from surgical resection specimens usually within 2 weeks after diagnosis. Of the 253 patients, 31 were excluded because of incomplete clinical data. In the remaining 222 patients, tumor samples obtained at surgery were histologically graded as well (G1), moderately (G2) differentiated, and poorly (G3) differentiated. Margins of the resected specimen and lymph nodes were examined by a histopathologist and judged for tumor involvement and 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. Surgically treated cases were seen as outpatients every 3–6 months for up to 5 years. The occurrence of second primary tumors was recorded.

**Identification of Genotypes.** Genotypes were identified in leukocyte DNA after PCR amplification (7, 9). *GSTM1 A*, *B*, *A/B*, and *null* were identified using allele-specific primers to exon 7. *GSTM3* genotypes were identified using primers to exon 6/7 (25). *GSTT1 null* and expressing subjects were also identified using PCR. The *GSTP1* $Ile^{105}/Ile^{105}$, $Ile^{105}/Val^{105}$, and $Val^{105}/Val^{105}$ genotypes were identified after PCR amplification and digestion with *Alw261*. Two mutant CYP2D6 alleles, *CYP2D6*4* (G→A transition at intron 3/exon 4) and *CYP2D6*3* (bp deletion in exon 5), were identified in separate PCR assays. Homozygotes or compound heterozygotes for these alleles were classed as PMs. Heterozygotes for either *CYP2D6*4* or *CYP2D6*3* and wild-type *CYP2D6*1* were classified as HET, whereas homozygotes for *CYP2D6*1* are EM. Some subjects classed as wild-type homozygotes could be heterozygotes for uncommon, variant alleles such as *CYP2D6*5* (gene deletion). Similarly, heterozygotes for *CYP2D6*4* or *CYP2D6*3* and *CYP2D6*5* were classed as PMs. Two *CYP1A1* mutations were identified (9): the 3′-flanking region mutation (*m1m1* wild-type homozygotes) and the exon 7 Ile>Val mutation (*Ile/Ile* wild-type homozygotes). The mutation in the *CYP2E1* 5′-flanking region containing the *Pst*I and *Rsa*I digestion sites (*c1c1* wild-type homozygotes) and *Dra*I RFLP (*DD* wild-type homozygotes) were also identified (9). The *CCND1* genotype was identified using amplification across the exon 4/intron 4 A/G polymorphism (7). PCR product was digested with *Scrf* I, and the *CCND1* genotype was assigned after visualization of 113-bp (*CCND1 AA*), 91-bp (*CCND1 GG*) or 113- and 91-bp (*CCND1 AG*) fragments. In some patients, genotyping data could not be obtained for all of

the genes under study. The missing data were not obtained because DNA samples were either exhausted or refractory to amplification. The missing samples were largely those obtained between 1994 and 1995 and were, therefore, lost at random and did not constitute a subgroup.

**Statistical Analysis.** Analyses were carried using Stata, version 5 (Stata Corporation, College Station, TX). We used a logistic regression model to examine the data for associations between individual genotypes and each outcome parameter. All end points were transformed to binary data (G1/G2 *versus* G3, T1/T2 *versus* T3, nodes$^-$ *versus* nodes$^+$). In all these analyses, the data were corrected for age at diagnosis, gender, and alcohol and smoking consumption because these factors are associated with outcome (1–4). In some cases, the data set was incomplete usually because DNA repeatedly failed to amplify in a particular genotyping assay or information on alcohol and tobacco consumption was not collected in busy clinics. Such failures occurred completely at random. In these instances, cases with missing data were excluded from the statistical analysis.

Our analytical strategy was as follows. First, in the main analysis, we studied associations between each gene individually and the three outcome parameters. These genes were selected for study on the basis of their presumed biological function. The reference categories were *GSTT1 A, GSTM1 null, GSTP1 Ile$^{105}$/Ile$^{105}$, GSTM3 AA, CYP2D6 EM, CYP2E1 c1c1, CYP2E1 DD, CYP1A1 m1m1, CYP1A1 Ile/Ile*, and *CCND1 AA*. For each gene, the factorized genotypes shown in Table 1 were studied. For information, we reported all associations, whether significant or not, between genotypes and outcome. We studied associations between eight genes (10 polymorphic loci) and three outcome parameters. Consequently, more than 100 significance tests were performed. We recognize that there are dangers in both making and not making inferences on individual factors in multiple testing (26, 27). We have presented only uncorrected *P*s because, using the Bonferroni procedure, none of the *P*s found to be $<0.05$ would remain significant. Indeed, the smallest uncorrected *P* of 0.002 (association of *CCND1 GG* with G3 tumors) would be $>0.20$ after correction. Next, in a second analysis, we used a step-wise routine ($P < 0.10$ for inclusion of a variable) to identify the best set of predictors for each outcome parameter. All of the genes were included in the step-wise routine together with age at diagnosis, gender, alcohol and tobacco consumption, and, tumor site. In this analysis, *CYP1A1 m1m2* and *m2m2* were combined because the frequency of *m2m2* was very low (Table 1). Similarly, *CYP1A1 Ile/Val* was combined with *Val/Val*, *CYP2E1 c1c2* was combined with *c2c2* and *CYP2E1 CD* with *CC*.

## Results

**Association between Clinical Outcome Measures.** We first examined the data on tumor extension, histological grade, and the presence of nodes to confirm expected associations between these outcome parameters. These were closely related; undifferentiated tumors (G3) were more likely to demonstrate lymph node involvement ($P < 0.0001$) even if initial surgical treatment was curative. Patients presenting with T3/T4 tumors were more likely to suffer lymph node metastasis ($P < 0.0001$) and undifferentiated tumors ($P < 0.0001$).

**Associations of Individual *GST*, *CYP*, and *CCND1* Genotypes with Tumor Extension.** Table 1 shows demographic data describing patient age at diagnosis, gender, and alcohol and tobacco consumption. The frequencies of *GST*, *CYP*, and *CCND1* genotypes in the total case group of 384 patients, with one SCCHN treated surgically and nonsurgically in whom associations between tumor extension (T1-T4) and genotypes could be assessed, are also shown in Table 1. As expected, the frequency of *GSTM1 AB* was lower in the cases (2.1%) than in German controls (7.3%; 9). Furthermore, the frequency of *GSTM3 AA* (76.6%) was higher than in controls (66.5%), whereas that of *GSTP1 Ile$^{105}$/Ile$^{105}$* was lower and that of *GSTP1 Ile$^{105}$/Val$^{105}$* higher than in controls (52.8% and 35.0%, respectively). The frequencies of the other genotypes in the cases were similar to those reported in controls (8, 9).

We used a logistic regression model (corrected for age at

*Table 1* Genotype frequencies in patients with one or more than one SCCHN

| | Single SCCHN ($n = 384$) | Multi-SCCHN ($n = 39$) |
|---|---|---|
| Age at diagnosis (yrs ± SD) | 60.4 ± 10.3 | 61.2 ± 6.8 |
| Males/females | 328/56 | 35/4 |
| Never smokers (%) | 29 (7.6) | 2 (5.1) |
| 1–10 cigarettes/day (%) | 32 (8.3) | 5 (12.8) |
| 11–20 cigarettes/day (%) | 186 (48.4) | 18 (46.2) |
| >20 cigarettes/day (%) | 120 (31.3) | 14 (35.9) |
| Missing data (%) | 17 (4.4) | |
| Never consumers of alcohol (%) | 50 (13.0) | 4 (10.3) |
| Up to 50 g/day (%) | 68 (17.7) | 7 (17.9) |
| 50–100 g/day (%) | 180 (46.9) | 22 (56.4) |
| >100 g/day (%) | 69 (18.0) | 6 (15.4) |
| Missing data | 17 (4.4) | |
| *GSTM* | | |
| *null* | 189 (56.4) | 23 (59.0) |
| *A* | 100 (29.8) | 10 (25.7) |
| *B* | 39 (11.6) | 4 (10.3) |
| *AB* | 7 (2.1) | 2 (5.1) |
| *GSTT1* | | |
| *null* | 69 (21.2) | 14 (36.8)[a] |
| *A* | 256 (78.8) | 24 (63.2) |
| *GSTM3* | | |
| *AA* | 255 (76.6) | 32 (82.1) |
| *AB* | 69 (20.7) | 7 (17.9) |
| *BB* | 9 (2.7) | 0 (0) |
| *GSTP1* | | |
| *Ile$^{105}$/Ile$^{105}$* | 149 (45.0) | 17 (44.7) |
| *Ile$^{105}$/Val$^{105}$* | 145 (43.8) | 18 (47.4) |
| *Val$^{105}$/Val$^{105}$* | 37 (11.1) | 3 (7.9) |
| *CYP2D6* | | |
| *EM* | 193 (58.1) | 23 (60.5) |
| *HET* | 115 (34.6) | 15 (39.5) |
| *PM* | 24 (7.2) | 0 (0) |
| *CYP1A1* | | |
| *m1m1* | 290 (86.6) | 35 (89.7) |
| *m1m2* | 44 (13.1) | 4 (10.3) |
| *m2m2* | 1 (0.3) | 0 (0) |
| *CYP1A1* | | |
| *Ile/Ile* | 281 (84.9) | 30 (76.9) |
| *Ile/Val* | 50 (15.1) | 8 (20.5) |
| *Val/Val* | 0 (0) | 1 (2.6) |
| *CYP2E1* | | |
| *c1c1* | 307 (94.2) | 35 (92.1) |
| *c1c2* | 18 (5.5) | 3 (7.9) |
| *c2c2* | 1 (0.3) | 0 (0) |
| *CYP2E1* | | |
| *DD* | 259 (86.3) | 32 (91.4) |
| *CD* | 41 (13.7) | 3 (8.6) |
| *CC* | 0 (0) | 0 (0) |
| *CCND1* | | |
| *AA* | 80 (23.4) | 7 (18.9) |
| *AG* | 177 (51.7) | 19 (51.4) |
| *GG* | 85 (24.9) | 11 (29.7) |

[a] Frequency of *GSTT1 null* in the cases with more than one SCC *versus* cases with one SCC; $P = 0.014$, OR = 2.47.

*Table 2* Association of genotypes (studied individually) on outcome parameters in SCCHN

A. Tumor extension: individual genes (corrected)

| | Outcome parameter | | $P^a$ | OR[b] | 95% CI |
|---|---|---|---|---|---|
| | T1/T2 | T3/T4 | | | |
| Total case group | | | | | |
| *GSTM1 A* | 53/190 (27.9) | 47/145 (32.4) | 0.75 | 1.09 | 0.65, 1.82 |
| *GSTM3 BB* | 3/187 (1.6) | 6/146 (4.1) | 0.37 | 1.92 | 0.46, 7.97 |
| *GSTT1 null* | 31/180 (17.2) | 38/145 (26.2) | 0.036 | 1.82 | 1.04, 3.19 |
| *GSTP1 Val/Val* | 22/184 (12.0) | 15/147 (10.2) | 0.83 | 0.92 | 0.43, 1.98 |
| *CYP2D6 PM* | 3/187 (1.6) | 6/146 (4.1) | 0.33 | 1.58 | 0.64, 3.92 |
| *CYP1A1 Ile/Val* | 12/188 (6.4) | 12/144 (8.3) | 0.54 | 1.22 | 0.63, 2.37 |
| *CYP1A1 m1m2* | 31/185 (16.8) | 19/146 (13.0) | 0.56 | 0.82 | 0.43, 1.58 |
| *CYP2E1 CD* | 24/163 (14.7) | 17/137 (12.4) | 0.80 | 0.91 | 0.44, 1.84 |
| *CYP2E1 c1c2* | 13/180 (7.2) | 5/146 (3.4) | 0.10 | 0.37 | 0.11, 1.21 |
| *CCND1 GG* | 53/194 (27.3) | 32/148 (21.6) | 0.31 | 0.70 | 0.36, 1.38 |
| Cases with oral/pharynx SCC | | | | | |
| *GSTM1 A* | 13/45 (17.8) | 21/64 (32.8) | 0.88 | 0.93 | 0.36, 2.38 |
| *GSTM3 BB* | 2/45 (4.4) | 3/65 (4.6) | 0.50 | 0.52 | 0.08, 3.47 |
| *GSTT1 null* | 7/40 (17.5) | 21/63 (33.3) | 0.029 | 3.43 | 1.13, 10.31 |
| *GSTP1 Val/Val* | 7/45 (15.6) | 8/65 (12.3) | 0.70 | 1.30 | 0.54, 3.42 |
| *CYP2D6 PM* | 3/43 (7.0) | 2/63 (3.2) | 0.84 | 1.10 | 0.44, 2.80 |
| *CYP1A1 Ile/Val* | 3/45 (6.7) | 7/65 (10.7) | 0.62 | 1.47 | 0.32, 6.70 |
| *CYP1A1 m1m2* | 5/45 (11.1) | 10/64 (15.6) | 0.70 | 1.29 | 0.35, 4.80 |
| *CYP2E1 CD* | 7/42 (16.7) | 8/63 (12.7) | 0.65 | 0.75 | 0.22, 2.56 |
| *CYP2E1 c1c2* | 2/44 (4.6) | 3/64 (4.7) | 0.93 | 1.09 | 0.16, 7.45 |
| *CCND1 GG* | 11/50 (22.0) | 13/67 (19.4) | 0.25 | 0.46 | 0.13, 1.70 |
| Cases with larynx SCC | | | | | |
| *GSTM1 A* | 40/146 (27.6) | 26/81 (32.1) | 0.60 | 1.18 | 0.62, 2.26 |
| *GSTM3 BB* | 1/142 (0.7) | 3/81 (3.7) | 0.21 | 4.32 | 0.43, 43.1 |
| *GSTT1 null* | 24/140 (17.1) | 17/82 (20.7) | 0.39 | 1.37 | 0.67, 2.79 |
| *GSTP1 Val/Val* | 15/139 (10.8) | 7/82 (8.5) | 0.40 | 0.65 | 0.24, 1.76 |
| *CYP2D6 PM* | 9/145 (6.2) | 10/81 (12.4) | 0.085 | 2.43 | 0.89, 6.72 |
| *CYP1A1 Ile/Val* | 20/142 (14.1) | 14/83 (16.9) | 0.57 | 1.25 | 0.58, 2.70 |
| *CYP1A1 m1m2* | 26/140 (18.6) | 9/82 (11.0) | 0.28 | 0.63 | 0.27, 1.45 |
| *CYP2E1 CD* | 17/121 (14.1) | 9/74 (12.2) | 0.80 | 0.88 | 0.35, 2.23 |
| *CYP2E1 c1c2* | 11/136 (8.1) | 2/82 (2.4) | 0.072 | 0.14 | 0.02, 1.19 |
| *CCND1 GG* | 42/144 (29.2) | 19/81 (23.5) | 0.98 | 0.99 | 0.42, 2.34 |

B. Histological differentiation: individual genes (corrected)

| | Outcome parameter | | $P^a$ | OR[b] | 95% CI |
|---|---|---|---|---|---|
| | G1/G2 | G3 | | | |
| Total case group | | | | | |
| *GSTM1 A* | 50/149 (33.6) | 13/50 (26.0) | 0.43 | 0.72 | 0.31, 1.63 |
| *GSTM3 BB* | 6/146 (4.1) | 0/52 (0) | 0.34 | –[c] | –[c] |
| *GSTT1 null* | 27/139 (19.4) | 16/48 (34.8) | 0.086 | 2.04 | 0.90, 4.58 |
| *GSTP1 Val/Val* | 13/145 (9.0) | 3/50 (6.0) | 0.83 | 1.15 | 0.27, 4.89 |
| *CYP2D6 PM* | 15/148 (10.1) | 4/49 (8.2) | 0.78 | 0.83 | 0.22, 3.03 |
| *CYP1A1 Ile/Val* | 20/147 (13.6) | 6/52 (11.5) | 0.66 | 1.26 | 0.44, 3.61 |
| *CYP1A1 m1m2* | 26/144 (18.1) | 7/50 (14.0) | 0.99 | 1.00 | 0.38, 2.61 |
| *CYP2E1 CD* | 20/130 (15.4) | 5/43 (11.6) | 0.84 | 1.12 | 0.36, 3.52 |
| *CYP2E1 c1c2* | 5/141 (3.6) | 1/50 (2.0) | 0.63 | 1.82 | 0.16, 21.27 |
| *CCND1 GG* | 36/160 (22.5) | 21/54 (38.9) | 0.002 | 8.44 | 2.17, 32.73 |
| Cases with oral/pharynx SCC | | | | | |
| *GSTM1 A* | 17/47 (36.2) | 7/30 (23.3) | 0.26 | 0.50 | 0.15, 1.68 |
| *GSTM3 BB* | 4/46 (8.7) | 0/32 (0) | 0.15 | –[c] | –[c] |
| *GSTT1 null* | 8/42 (19.1) | 12/26 (46.2) | 0.069 | 3.28 | 0.91, 11.86 |
| *GSTP1 Val/Val* | 5/46 (10.9) | 1/31 (3.2) | 0.95 | 1.10 | 0.07, 18.32 |
| *CYP2D6 PM* | 3/45 (6.7) | 2/29 (6.9) | 0.78 | 1.42 | 0.12, 17.17 |
| *CYP1A1 Ile/Val* | 6/46 (13.0) | 3/32 (9.4) | 0.86 | 0.86 | 0.16, 4.57 |
| *CYP1A1 m1m2* | 7/46 (15.2) | 7/30 (23.3) | 0.32 | 1.96 | 0.53, 7.28 |
| *CYP2E1 CD* | 5/44 (11.4) | 3/26 (11.5) | 0.65 | 1.50 | 0.25, 8.95 |
| *CYP2E1 c1c2* | 0/44 (0) | 0/31 (0) | –[d] | –[d] | –[d] |
| *CCND1 GG* | 10/54 (18.5) | 12/34 (35.3) | 0.011 | 11.09 | 1.73, 70.99 |

*Table 2* Continued

B. Tumor extension: individual genes (corrected)

| | Outcome parameter | | $P^a$ | OR[b] | 95% CI |
|---|---|---|---|---|---|
| | G1/G2 | G3 | | | |
| Cases with larynx SCC | | | | | |
| *GSTM1 A* | 33/102 (32.4) | 6/20 (30.0) | 0.72 | 0.79 | 0.23, 2.75 |
| *GSTM3 BB* | 2/100 (2.0) | 0/20 (0) | 1.00 | –[c] | –[c] |
| *GSTT1 null* | 19/97 (19.6) | 4/20 (20.0) | 0.92 | 1.06 | 0.29, 3.87 |
| *GSTP1 Val/Val* | 8/99 (8.1) | 2/19 (10.5) | 0.97 | 1.03 | 0.17, 6.45 |
| *CYP2D6 PM* | 12/103 (11.7) | 2/20 (10.0) | 0.96 | 0.96 | 0.17, 5.38 |
| *CYP1A1 Ile/Val* | 14/101 (13.9) | 3/20 (15.0) | 0.57 | 1.53 | 0.36, 6.50 |
| *CYP1A1 m1m2* | 19/98 (19.4) | 0/20 (0) | 0.040 | –[c] | –[c] |
| *CYP2E1 CD* | 15/86 (17.4) | 2/17 (11.8) | 0.86 | 0.85 | 0.15, 4.67 |
| *CYP2E1 c1c2* | 5/97 (5.2) | 1/19 (5.3) | 0.39 | 3.00 | 0.24, 36.92 |
| *CCND1 GG* | 26/106 (24.5) | 9/20 (45.0) | 0.042 | 9.86 | 1.09, 89.09 |

C. Nodes: individual genes (corrected)

| | Outcome parameter | | $P^a$ | OR[b] | 95% CI |
|---|---|---|---|---|---|
| | $Nodes^-$ | $Nodes^+$ | | | |
| Total case group | | | | | |
| *GSTM1 A* | 33/119 (27.7) | 31/81 (38.3) | 0.078 | 1.86 | 0.93, 3.72 |
| *GSTM3 BB* | 4/117 (3.4) | 2/82 (2.4) | 0.70 | 0.70 | 0.12, 4.07 |
| *GSTT1 null* | 20/111 (18.0) | 20/75 (26.7) | 0.29 | 1.50 | 0.71, 3.23 |
| *GSTP1 Val/Val* | 11/113 (9.7) | 7/83 (8.4) | 0.65 | 0.76 | 0.23, 2.49 |
| *CYP2D6 PM* | 8/119 (6.7) | 10/79 (12.7) | 0.035 | 3.33 | 1.09, 10.16 |
| *CYP1A1 Ile/Val* | 16/117 (13.7) | 9/83 (10.8) | 0.99 | 1.00 | 0.38, 2.61 |
| *CYP1A1 m1m2* | 19/113 (16.8) | 13/82 (15.9) | 0.52 | 0.75 | 0.31, 1.80 |
| *CYP2E1 CD* | 17/99 (17.2) | 7/76 (9.2) | 0.53 | 0.72 | 0.26, 2.00 |
| *CYP2E1 c1c2* | 5/111 (4.5) | 0/81 (0) | 0.075 | –[c] | –[c] |
| *CCND1 GG* | 30/120 (25.0) | 27/93 (29.0) | 0.49 | 1.37 | 0.55, 3.40 |
| Cases with oral/pharynx SCC | | | | | |
| *GSTM1 A* | 7/26 (26.9) | 19/52 (36.5) | 0.55 | 1.41 | 0.45, 4.42 |
| *GSTM3 BB* | 2/26 (7.7) | 2/53 (3.8) | 0.43 | 0.42 | 0.05, 3.63 |
| *GSTT1 null* | 6/22 (27.3) | 12/47 (25.5) | 0.98 | 1.01 | 0.29, 3.53 |
| *GSTP1 Val/Val* | 2/25 (8.0) | 5/53 (9.4) | 0.80 | 1.38 | 0.11, 17.59 |
| *CYP2D6 PM* | 1/26 (3.9) | 4/49 (8.2) | 0.52 | 2.29 | 0.18, 28.68 |
| *CYP1A1 Ile/Val* | 4/26 (15.4) | 4/53 (7.6) | 0.50 | 0.55 | 0.10, 3.06 |
| *CYP1A1 m1m2* | 4/25 (16.0) | 8/52 (15.4) | 0.95 | 0.96 | 0.22, 4.11 |
| *CYP2E1 CD* | 4/24 (16.7) | 4/48 (8.3) | 0.95 | 0.94 | 0.15, 6.07 |
| *CYP2E1 c1c2* | 0/25 (0) | 0/51 (0) | –[d] | –[d] | –[d] |
| *CCND1 GG* | 6/28 (21.4) | 16/60 (26.7) | 0.32 | 2.19 | 0.50, 10.24 |
| Cases with larynx SCC | | | | | |
| *GSTM1 A* | 26/93 (28.0) | 12/29 (41.4) | 0.11 | 2.28 | 0.84, 6.21 |
| *GSTM3 BB* | 2/91 (2.2) | 0/29 (0) | 1.00 | –[c] | –[c] |
| *GSTT1 null* | 14/89 (15.7) | 8/28 (28.6) | 0.084 | 2.60 | 0.88, 7.66 |
| *GSTP1 Val/Val* | 9/88 (10.2) | 2/30 (6.7) | 0.29 | 0.39 | 0.07, 2.20 |
| *CYP2D6 PM* | 7/93 (7.5) | 6/30 (20.0) | 0.013 | 6.49 | 1.48, 28.30 |
| *CYP1A1 Ile/Val* | 12/91 (13.2) | 5/30 (16.7) | 0.45 | 1.60 | 0.47, 5.52 |
| *CYP1A1 m1m2* | 15/88 (17.1) | 5/30 (16.7) | 0.48 | 0.61 | 0.16, 2.40 |
| *CYP2E1 CD* | 13/75 (17.3) | 3/28 (10.7) | 0.70 | 0.75 | 0.18, 3.16 |
| *CYP2E1 c1c2* | 5/86 (5.8) | 0/30 (0) | 0.33 | –[c] | –[c] |
| *CCND1 GG* | 24/92 (26.1) | 11/33 (33.3) | 0.72 | 1.27 | 0.34, 4.74 |

[a] Factorized genotype frequencies were compared in cases with T1/T2 *versus* T3/T4 tumors, cases with G1/G2 *versus* G3 tumors, and in patients with and without nodes. Logistic regression analysis, corrected for age at diagnosis, gender, and alcohol and smoking consumption was used.
[b] Reference categories (OR = 1) were: *GSTT1 A, GSTM1 null, GSTM3 AA, GSTP1* $Ile^{105}/Ile^{105}$, *CYP2D6 EM, CYP2E1 c1c1, CYP2E1 DD, CYP1A1 m1m1, CYP1A1 Ile/Ile, CCND1 AA.*
[c] Unable to calculate OR due to zero values in one category. *P* presented as uncorrected data from Fishers' exact test.
[d] Unable to calculate OR or *P* due to zero values in both categories.

diagnosis, gender, alcohol and smoking consumption) to examine the data for associations between tumor extension and the factorized genotypes, as shown in Table 1, for each individual gene. In the total case group, only *GSTT1 null* was significantly associated ($P = 0.036$) with T3/T4 tumors (Table 2). *CYP2E1 c1c2* was associated with T1/T2 tumors, although the association only approached significance ($P = 0.10$). In the oral cavity/pharyngeal cases, the frequency of *GSTT1 null* was significantly higher in the cases with T3/T4 tumors than in those with T1/T2 tumors ($P = 0.029$; Table 2). There were no significant associations between any of the genotypes and tumor extension in the laryngeal SCC cases, although the associations with *CYP2E1 c1c2* and *CYP2D6 PM* approached significance ($P = 0.072$ and $P = 0.085$, respectively). *CYP2E1*

*Table 3* Associations between combinations of *GSTT1* and *CCND1* genotypes and histological grade

| | Total SCCHN group | | | | Oral/pharynx SCC | | | | Larynx SCC | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *P* | OR | 95% CI | *n* | *P* | OR | 95% CI | *n* | *P* | OR | 95% CI | *n* |
| *GSTT1 A/CCND1 AA* | Reference category | | | 31 | Reference category | | | 12 | Reference category | | | 19 |
| *GSTT1 null/CCND1 AA* | 0.56 | 2.18 | 0.16, 30.0 | 8 | 0.39 | 4.00 | 0.16, 96.2 | 3 | 1.00 | –[a] | | 5 |
| *GSTT1 A/CCND1 AG* | 0.16 | 3.12 | 0.64, 15.2 | 73 | 0.22 | 4.11 | 0.43, 39.7 | 26 | 0.37 | 2.89 | 0.29, 29.0 | 47 |
| *GSTT1 null/CCND1 AG* | 0.021 | 7.89 | 1.36, 45.7 | 22 | 0.12 | 8.47 | 0.56, 127.2 | 10 | 0.14 | 6.42 | 0.54, 76.5 | 12 |
| *GSTT1 A/CCND1 GG* | 0.011 | 8.45 | 1.64, 43.6 | 36 | 0.083 | 9.07 | 0.75, 109.8 | 10 | 0.072 | 7.85 | 0.83, 74.4 | 26 |
| *GSTT1 null/CCND1 GG* | 0.010 | 11.6 | 1.78, 75.2 | 12 | 0.012 | 35.66 | 2.20, 577.8 | 7 | 0.38 | 3.86 | 0.18, 81.3 | 5 |

[a] Unable to calculate OR due to zero values in one category. *P* presented as uncorrected data from Fisher's exact test.

*c1c2* was associated with T1/T2 tumors. The association of *GSTT1 null* with tumor extension was not significant, although the OR was >1.

**Associations of Individual *GST*, *CYP*, and *CCND1* Genotypes with Histological Differentiation.** We, again, used a logistic regression model to identify associations between the factorized genotypes and differentiation (G1-G3). Table 2 shows that in the surgically treated patients with a single SCCHN, only *CCND1 GG* was significantly associated ($P = 0.002$) with poorly differentiated tumors (G3), although the association with *GSTT1 null* approached significance. In the oral cavity/pharyngeal cases, the frequency of *CCND1 GG* was significantly higher in the cases with G3 than in those with G1/G2 tumors (Table 2). Although *GSTP1 $Val^{105}/Val^{105}$* was not associated with histological differentiation (Table 2), *GSTP1 $Ile^{105}/Val^{105}$* was significantly associated with G3 tumors ($P = 0.018$, OR 4.51, 95% CI 1.29, 15.79). The association of *GSTT1 null* with G3 tumors did not quite achieve significance ($P = 0.069$). In the laryngeal SCC cases, *CCND1 GG* was significantly associated with histological differentiation. *CYP1A1 m1m2* was significantly associated with G!/G2 tumors, although this analysis was based on data that was uncorrected for age at diagnosis, gender, and alcohol and tobacco consumption.

Because the results shown in Table 2 suggest associations between histological grade and *GSTT1 null*, *GSTP1 $Ile^{105}/Val^{105}$*, and *CCND1 GG*, we examined the data for associations between grade and combinations of these genotypes. Table 3 shows that combination of *CCND1 GG* and *GSTT1 null*, *CCND1 GG* and *GSTT1 A*, and *CCND1 AG* and *GSTT1 null* were significantly associated with G3 tumors. The greatest quantitative effect, on the basis of the OR, was with *CCND1 GG* and *GSTT1 null*, although it should be noted that the 95% CI for the three genotype combinations overlap (Table 3). Similarly, in the oral/pharyngeal SCC cases, the *CCND1 GG/GSTT1* null combination was more strongly associated with G3 tumors than the others. No significant associations were observed between the genotype combinations and histological grade in the laryngeal SCC cases (Table 3). No significant associations between combinations of *GSTP1* and *CCND1* genotypes were observed.

**Associations of Individual *GST*, *CYP*, and *CCND1* Genotypes with Lymph Nodes.** Table 2 shows that in the patients with one SCCHN, only *CYP2D6 PM* was significantly associated ($P = 0.035$) with the presence of nodes, although the association with *GSTM1 A* approached significance ($P = 0.078$). The association between *CYP2E1 c1c2* and absence of nodes also approached significance, although this analysis was based on data that was uncorrected for age at diagnosis, gender, and alcohol and tobacco consumption. None of the genotypes were associated with lymph node involvement in the oral/pharyngeal SCC cases. In the laryngeal SCC cases, *CYP2D6 HET* ($P = 0.020$, OR 3.40, 95% CI 1.21, 9.55) and *CYP2D6 PM* were significantly associated with the presence of nodes and the association with *GSTT1 null* and *GSTP1 $Ile^{105}/Val^{105}$* ($P = 0.085$, OR 0.42, 95% CI 0.16, 1.13) approached significance. *GSTP1 $Val^{105}/Val^{105}$* was not associated with the presence of nodes.

*Table 4* Use of a step-wise model to study associations of genotypes with outcome parameters in SCCHN

| | *P* | OR | 95% CI |
|---|---|---|---|
| Tumor extension ($n = 268$) | | | |
| Oral cavity/pharyngeal site[a] | 0.001 | 2.44 | 1.45, 4.17 |
| Ethanol consumption | 0.003 | 2.36 | 1.34, 4.15 |
| *GSTT1 null* | 0.063 | 1.81 | 0.97, 3.39 |
| *CYP2E1 c1c2* and *c2c2* | 0.081 | 0.31 | 0.08, 1.16 |
| Histological differentiation ($n = 143$) | | | |
| Oral cavity/pharyngeal site[a] | 0.032 | 2.44 | 1.08, 5.26 |
| *CCND1 GG* | 0.041 | 2.44 | 1.04, 5.75 |
| *GSTT1 null* | 0.100 | 2.08 | 0.87, 4.97 |
| Presence of nodes ($n = 141$) | | | |
| Oral cavity/pharyngeal site[a] | <0.001 | 5.55 | 2.56, 12.50 |
| *CYP2D6 PM* | 0.006 | 6.99 | 1.75, 27.89 |
| *GSTM1 null* | 0.049 | 0.46 | 0.22, 1.00 |
| Tobacco consumption | 0.066 | 3.03 | 0.93, 9.90 |

[a] The step-wise model included *GSTM1 null*, *GSTT1 null*, *GSTM3 BB*, *GSTP1 $Val^{105}/Val^{105}$*, *CYP2D6 PM*, *CYP1A1 Ile/Val* and *Val/Val*, *CYP1A1 m1m2* and *m2m2*, *CYP2E1 c1c2* and *c2c2*, *CYP2E1 CD* and *DD*, *CCND1 GG*, and age at diagnosis, gender, alcohol and smoking consumption, and tumor site.

**Step-Wise Analysis.** We used a step-wise model to determine the relative influence of the genotypes in predicting each of the outcome parameters. This analysis was carried out in the total case group available for each outcome parameter and not in the cases subdivided by tumor site. This limitation was necessary because not all of the patients shown in Table 2 had complete data sets and some of the subgroups of patients with all of the relevant information were rather small ($n < 70$ patients). The model included: *GSTM1 null*; *GSTT1 null*; *GSTM3 BB*; *GSTP1 $Val^{105}/Val^{105}$; CYP2D6 PM*; *CYP1A1 Ile/Val* and *Val/Val*; *CYP1A1 m1m2* and *m2m2*; *CYP2E1 c1c2* and *c2c2*; *CYP2E1 CD* and *DD*; *CCND1 GG*; and age at diagnosis, gender, alcohol and tobacco consumption, and tumor site. In the total case group comprising surgically and nonsurgically treated cases, a complete data set was available in 268 patients. In this group, the association of tumor extension with *GSTT1 null* ($P = 0.063$) and *CYP2E1 c1c2* and *c2c2* ($P = 0.081$) approached significance (Table 4). Ethanol consumption and oral cavity/pharyngeal SCC were associated with T3/T4 tumors ($P = 0.003$ and $P = 0.001$ respectively). In the surgically treated group, *CCND1 GG* was the only genotype that was signifi-

cantly associated with poor histological differentiation ($P = 0.041$), although the association with *GSTT1 null* approached significance ($P = 0.100$). Oral cavity/pharyngeal SCC were also significantly associated with G3 tumors. We next studied the influence of genotypes in a model predicting involvement of nodes. *CYP2D6 PM* and *GSTM1 null* were associated with nodes ($P = 0.006$ and $P = 0.049$), together with tobacco consumption ($P = 0.066$). Importantly, the OR for the association between *GSTM1 null* and nodes was 0.48, indicating that this genotype is protective (Table 4). Oral cavity/pharyngeal SCC were also significantly associated with node involvement ($P < 0.001$).

***GST* Genotypes in Cases with More Than One SCCHN.** Table 1 shows the frequencies of the *GST*, *CYP*, and *CCND1* genotypes in the cases with more than one SCCHN. The *GSTT1 null* frequency was greater in patients with second primary tumors than in those with a single lesion ($P = 0.014$, OR = 2.47).

## Discussion

The factors that determine outcome in SCCHN remain unclear. Although the importance of tobacco and alcohol consumption as risk factors suggests that genes encoding detoxifying enzymes are susceptibility candidates, some data have not confirmed reported associations. Recent studies suggest that various *GST* and *CYP* genotypes are linked with outcome rather than susceptibility (7, 16, 23). Thus, the results obtained in population studies may reflect differences in the clinical stage of cases rather than differences between affected and unaffected individuals. Accordingly, we have determined whether *GST*, *CYP*, and *CCND1* genotypes are associated with outcome in SCCHN.

The natural history of malignancies is considered to involve malignant change in the target cell, growth of transformed cells, local invasion, and metastasis. Thus, differentiation describes the extent that tumor cells morphologically and functionally resemble normal cells. Metastases are a marker of malignancy, although cancers are heterogeneous with regard to metastatic potential. This heterogeneity may result from new mutations in genetically unstable cells or variable expression of key genes in different subsets of tumor cells (28). As expected, therefore, although the outcome parameters studied were significantly associated (1, 3, 28), they were not similarly associated with genetic factors. Some of these parameters also depend, to different degrees, on patient compliance and treatment modality. Thus, histological differentiation is not influenced by patient behavior. Lymph node involvement is also more frequent in patients presenting with late stage disease, but when found in small primary tumors or glottic laryngeal tumors it reflects the aggressiveness of the malignancy. Initial tumor extension is most likely to be influenced by patient behavior because almost all tumors will extend to T4 if diagnosis is delayed. Furthermore, the association of genotypes with outcome is likely to vary in different sites. Thus, glottic laryngeal SCC is usually diagnosed in its early stages before lymph node metastasis, whereas pharyngeal SCC cause symptoms only in progressed stages when lymph node involvement is usual (1, 2). An additional outcome parameter, the development of a second primary tumor is less influenced by patient compliance, although continued tobacco and alcohol consumption increases the risk of further SCCHN (24). These patients are of much interest because links between allelic variants and susceptibility/outcome are likely to be most evident in high-risk subgroups (23).

In the main analysis, we identified individual genes that were associated with the outcome parameters in the different sites. Thus, *GSTT1 null* was associated with T3/T4 lesions in the oral/pharyngeal, but not laryngeal, SCC cases. *GSTT1 null* was also associated with G3 lesions in the oral/pharyngeal, but not laryngeal, SCC cases. This, association was not significant, although the values of the ORs were similar to those found for the association with T3/T4 lesions. *CCND1 GG* was also associated with G3 tumors in the oral cavity/pharyngeal and laryngeal SCC cases. The link between *GSTT1 null*, *CCND1 GG*, and histological grade was also demonstrated by the significant association, in the oral/pharyngeal SCC cases, between this genotype combination and G3 tumors. In a secondary analysis, we used a step-wise routine to obtain the best set of predictors for each outcome parameter and to allow assessment of each genotype within this set. *GSTT1 null*, *CCND1 GG*, and *CYP2D6 PM* were associated with the appropriate parameter in this routine. The importance of *GSTT1 null* was demonstrated by the finding that its frequency was higher in cases with more than one primary lesion than in those with one tumor.

Associations between outcome parameters and other genes were also significant or approached significance. *CYP2E1 c1c2* was associated with T1/T2 tumors in the laryngeal SCC cases and, in the step-wise analysis, *CYP2E1 c1c1* was associated with T3/T4 lesions. These data suggest that the *c2* allele is protective. *CYP1A1*, although a good candidate, was not associated with tumor extension or the presence of nodes. The association of *CYP1A1 m1m2* with histological differentiation in laryngeal SCC cases was significant, although the analysis was based on uncorrected data. This and other *CYP* genes are difficult to study in Caucasians because the frequency of mutant alleles is generally low (9, 10, 12). We did not identify any significant associations between *GSTP1* $Val^{105}/Val^{105}$ and the outcome parameters. We did, however, identify contrasting associations between *GSTP1* $Ile^{105}/Val^{105}$ and tumor differentiation and the presence/absence of nodes. Thus, the heterozygote genotype was significantly associated with G3 tumors in oral/pharyngeal SCC cases and with the absence of nodes in the laryngeal SCC cases, although this link did not achieve significance. The mechanism for these apparently site-specific associations of the genotype is unknown, although we have reported in these patients that the *GSTP1* $Ile^{105}/Ile^{105}$ is associated with susceptibility to oral/pharyngeal, but not laryngeal, SCC (8). The finding that *GSTP1* genotypes are not associated with laryngeal SCC has been confirmed (17). Furthermore, the frequency of the *GSTP1* $Ile^{105}/Ile^{105}$ was lower and that of *GSTP1* $Ile^{105}/Val^{105}$ was higher in cases with oral/pharyngeal SCC than in controls (9). This suggests the $Val^{105}$ allele is associated with risk of less differentiated lesions in this site. However, the basis for the association of the clinical phenotype with a heterozygote, but not a homozygote, genotype is not known. Associations between *GSTM1* genotypes and outcome were also identified. Whereas most studies have considered *GSTM1*0* homozygotes to be at risk, the few studies that have also identified *GSTM1 AB*, *GSTM1 A*, and *GSTM1 B* suggest these expressed genotypes are not equally protective (15). Thus, in SCCHN cases, *GSTM1 AB*, but not *GSTM1 A* or *GSTM1 B*, is protective (9). Indeed, in some other cancers, *GSTM1 B* is associated with increased risk compared with *GSTM1 null* (23). Similarly, in this study *GSTM1 null* was associated with the absence of nodes in the step-wise routine. These data may reflect linkage dysequilibrium between the $\mu$ *GST* genes. Thus, *GSTM1*A* is in linkage dysequilibrium with *GSTM3*B* (25). However, although the association between *GSTM1 A* and nodes approached significance, we did not identify any links

between *GSTM1 B* and outcome. We also did not identify any links between *GSTM3* and the outcome parameters studied.

The mechanism for the association of *GST* and *CYP* genotypes on outcome presumably reflects their influence on the detoxication of potential mutagens, a key factor in SCCHN (29). Indeed, associations between allelic variants and the formation of mutations in key target genes have been reported in several cancers. Thus, *GSTM1 null* lung cancer patients demonstrate an increased frequency of p53 and H-ras-1 mutations and ovarian cancer patients with this genotype or *GSTT1 null* demonstrate overexpression of p53 (30, 31). Importantly, chronic tobacco and alcohol consumption is associated with a high frequency of p53 mutations in tumors from SCCHN cases (32). Loss of function of p53 should lead to clonal expansion of a population of cells with a selective growth advantage. This will lead to further genetic changes in the dedifferentiating tissue predisposing to tumor progression, lymph node metastasis and a worse prognosis (32). A further related possibility is that *GST* genes, particularly *GSTT1*, influence genomic stability. Thus, *GSTT1 null* is linked with a higher background rate of sister chromatid exchange, possibly because of less efficient detoxication (33). Indeed, ineffective DNA repair seems to be an important factor in SCCHN, with recent studies showing reduced expression of *hMLH1* and *hGTBP/hMSH6* in lymphocytes from cases (34). The association of *CYP2D6 PM* and *HET* with laryngeal, but not oral/pharyngeal, SCC complements data showing these genotypes are linked with the time to cervical lymph node metastases in oral cavity SCC patients (16). The mechanism for this observation is unknown. C*YP2D6* does not seem to be strongly expressed in these tissues and, typically for detoxifying enzymes, the *in vivo* substrate is not known. (14, 15). Furthermore, it is important to recognize that lymph node involvement in these anatomical sites reflects differences in tumor behavior; in glottic laryngeal SCC, the presence of nodes is associated with aggressive tumors in late-stage disease, whereas it is a common finding in early pharyngeal cancer. We did not find our previously reported association between *CYP2D6 PM* and time to cervical node metastases (16), presumably because the number of German oral cavity SCC cases in this study was relatively small ($n = 38$). *CCND1 GG* was associated with G3 tumors. The mechanism for this association is also unknown, although because the *G* allele splices less of the *b* transcript than the *A* allele, individuals with *CCND1 GG* may have different cellular levels of cyclin D1 protein to subjects with *CCND1 AA*. Such differences in level may be critical because cells overexpressing this protein demonstrate loss of control of the $G_1$-S checkpoint. Furthermore, the ability of cyclin D1 to enhance gene amplification may encourage genetic instability (35), a characteristic of SCCHN (34).

The genetic basis of complex traits such as susceptibility and outcome is unclear (36–38). Although they must be determined by multiple loci, the number of genes involved is unknown, as is the nature of the relationships between risk and protective alleles. We have studied associations between eight genes and three outcome measures in different sites in the upper aerodigestive tract. Our data have identified associations between *CYP2D6 PM* and *GSTT1 null* and outcome that are compatible with studies in other patient groups (5, 16). Furthermore, *CCND1 GG* is associated with poorly differentiated lesions. Such tumors often demonstrate a large number of mitoses, a phenomenon compatible with deregulated expression of *CCND1*. Studies involving a large number of variables require the performance of many significance tests. Clearly, in our study, some of the observed significant (or near significant) associations could arise from multiple testing. Indeed, if we had included correction for multiple testing (26), none of the observed associations would remain significant. To obtain significant results after such correction would require thousands of cases. Importantly, Perneger (27) has argued that correction for multiple testing is inappropriate because it increases the likelihood of type II errors. Clearly, this issue presents problems for future studies identifying associations between clinical phenotypes and increasingly large numbers of genes. Indeed, recent studies emphasize the importance of epistasis (36–39). Epistasis describes a phenotype that results from the interaction between two or more genes that could not be predicted from the sum of the effects of the separate genes. Inclusion of such interactions in molecular epidemiological studies will inevitably result in even larger numbers of significance tests.

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