Serologic Evidence of Herpes Simplex Virus 1 Infection and Oropharyngeal Cancer Risk

Jacqueline R. Starr, Janet R. Daling, E. Dawn Fitzgibbons, Margaret M. Madeleine, Rhoda Ashley, Denise A. Galloway, and Stephen M. Schwartz

Department of Epidemiology, School of Public Health and Community Medicine, University of Washington, Seattle, WA 98195 [J. R. S., J. R. D., M. M. M., S. M. S.]; Program in Epidemiology, Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98109 [J. R. D., E. D. F., M. M. M., S. M. S.]; Department of Laboratory Medicine, School of Medicine, University of Washington, Seattle, WA 98195 [R. A.]; Program in Cancer Biology, Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98109 [D. A. G.]; Department of Microbiology, School of Medicine, University of Washington, Seattle, WA 98195 [D. A. G.]

ABSTRACT

In vitro and animal models suggest that the herpes simplex virus 1 (HSV1) may contribute to the development of oropharyngeal squamous cell carcinoma (OSCC). To determine whether the risk of OSCC is related to infection with HSV1 in humans, we recruited 260 patients from 18 to 65 years old who were newly diagnosed with OSCC between 1990–1995 while residing in three western Washington State counties. For comparison, we recruited at random 445 controls frequency matched to cases on age and sex. Participants completed in-person interviews and provided serum samples that were tested for antibody response to HSV1. After adjusting for sex, cigarette smoking, alcohol consumption, age, and income, HSV1 antibody positivity was associated with a slightly increased risk of OSCC [adjusted odds ratio (OR), 1.3; 95% confidence interval (CI), 0.9–2.0]. The adjusted association between HSV1 antigen positivity and OSCC risk among those who were current cigarette smokers (OR, 4.2; CI, 2.4–7.1) was stronger than would be predicted based on the additive combination of smoking alone (OR, 2.3; CI, 1.2–4.2) and HSV1 seropositivity alone (OR, 1.0; CI, 0.6–1.7). There was suggestive evidence that the association between HSV1 infection and OSCC was similarly modified by evidence of HPV infection but no evidence of effect modification with alcohol consumption. This population-based study suggests that HSV1 may enhance the development of OSCC in individuals who are already at increased risk of the disease because of cigarette smoking or HPV infection.

INTRODUCTION

HSV1 is a large, double-stranded DNA virus that predominantly infects the oral mucosa and causes oral “cold” sores. HSV1 lives latently in the neurons of infected individuals and is thought to reactivate regularly and asymptotically (1–6). The persistence of the virus in the oral cavity and its ability to stimulate host DNA synthesis and repair during reactivations suggest that it may contribute to OSCC development. Indeed, in vitro studies have elucidated specific mechanisms through which HSV1 may induce the transformation of human cells: HSV1 infection of human cell cultures has been shown to be mutagenic (7–9), to induce DNA synthesis (7, 8), and to inhibit apoptosis (3, 10), all of which may contribute to carcinogenesis (11, 12). Animal experiments also support the hypothesis that HSV1 contributes to tumor development (7, 13).

Despite the suggestive findings among laboratory studies, it is unclear whether HSV1 contributes to the OSCC risk in human populations. Early epidemiological studies showed OSCC cases to have higher levels of HSV1 antibodies than healthy controls (14, 15). In contrast, two more recent case-control studies, including one from our group, found no differences in HSV1 seropositivity between OSCC cases and matched healthy controls (16, 17). Few investigators have reported on interactions between HSV1 and nonviral risk factors, such as cigarette smoking and alcohol use, or between HSV1 and other suspected viral risk factors such as HPV. The basic science literature suggests, however, that HSV1 may particularly influence OSCC development when other risk factors are present (7, 8, 13, 18–23). We report the results of a large, population-based case-control investigation into the association between OSCC and HSV1 infection.

PATIENTS AND METHODS

Subject Eligibility, Identification, and Recruitment. The details of the study design and recruitment have been reported previously (24). Using the files of the Cancer Surveillance System, a participant in the National Cancer Institute’s Surveillance, Epidemiology, and End Results program, we identified all male and female patients diagnosed with incident histologically confirmed OSCC between 1990–1995. Eligible case subjects were those 18–65 years old residing in King, Pierce, and Snohomish counties in Washington State. Tumor sites included the tongue, gum, floor of mouth, other, and unspecified parts of the mouth, tonsils, and oropharynx.

We used RDD to identify as eligible control subjects residents of King, Pierce, and Snohomish counties in Washington State who were 18–65 years old between 1990–1995, and who did not have a history of OSCC. Control subjects were frequency-matched to the sex and age distribution (18–19, 20–24, and 5-year age categories until 60–65) of the case subjects, with three controls matched to every two cases.

The overall response rate for case subjects was 63.3%, or 284 participants. The overall response rate for controls, calculated as the product of the RDD household screening rate and the interview participation rate, was 60.9%, or 477 participants.

The protocol for recruitment of case and control subjects was approved by the institutional review board of the Fred Hutchinson Cancer Research Center.

Data Collection. All data collection activities were conducted following written informed consent from each case or control subject.

Interviews. Case and control subjects participated in structured in-person interviews, during which all questions were directed toward the time period prior to each participant’s reference date. The reference date for a case subject was the month and year he or she was diagnosed with OSCC. The reference date for a particular control was assigned at random from among the possible case subject diagnosis dates that had occurred prior to the selection of the control through RDD.

Demographic characteristics ascertained included marital status as of the reference date, race, highest level of school attended, and combined family income in the reference year. As part of a large series of questions on health history each participant was asked, “have you ever been diagnosed with genital herpes?” If the study participant answered “yes,” the interviewer proceeded to ask how many episodes the participant had experienced, the site(s) of the infection, and whether the genital herpes was treated.

Cigarette smoking data included whether a person had ever smoked >100 cigarettes, and if so, details regarding continuous periods (episodes) during which smoking habits (e.g., packs per day) were relatively unchanged. Episodes were delineated according to the ages at which each case or control subject reported major changes in smoking habits. Case and control subjects were also asked whether or not they had used smokeless tobacco (chewing
tobacco, snuff, or mini-pouches), cigars, or pipes. We ascertained each participant’s history of alcohol consumption using an approach similar to that for ascertaining cigarette smoking history. Thus, among participants who reported having had at least four alcoholic beverages in any one year, we elicited separate age-defined periods in which alcohol consumption (e.g., the frequency of drinking and number of drinks) was relatively unchanged.

**Biological Specimens.** Each participating case and control subject was asked to provide a venous blood sample. Aliquots of serum were stored at −70°C until they were thawed for assays of HSV and HPV antibody response. In addition, we collected a sample of exfoliated oral tissue by using a soft-bristled toothbrush. Exfoliated cells from the inside of the upper and lower lips, the left and right sides of the hard palate, buccal mucosa, top and bottom of the tongue, and the surface of the gingiva were combined and then frozen at −70°C (24).

Among those men and women who participated in the in-person interview, we obtained blood specimens from 260 (91.5%) of 284 case subjects and 445 (93.3%) of 477 control subjects. We obtained exfoliated oral tissue for 226 (87%) of those 260 case subjects and 412 (92.6%) of 445 control subjects who could also be HSV serotyped. Blood and exfoliated oral tissue were collected at the time of the in-person interviews (the median time following diagnosis was 8 months). We obtained tumor tissue specimens from the case subjects as described previously (24), successfully testing 195 of 260 (75%) participants for evidence of HPV DNA.

**Laboratory Methods.** Previously unthawed serum aliquots from case and control subjects were provided to the laboratory in random order, blinded as to participant characteristics, and analyzed as to HSV antibody response using a single batch of HSV1- or HSV2-infected cell lysate. The Western blot method we used to determine HSV antibody status has been described elsewhere (25). Briefly, 5 μL of participants’ sera in 1 mL of BLOTTO were incubated separately with Western blot strips containing HSV1- or HSV2-infected cell proteins. In each set of samples, HSV1 and HSV2 strips were also incubated with control serum samples, which included HSV1-positive serum, HSV2-positive serum, HSV1- and HSV2-positive serum, HSV1- and HSV2-negative serum, and a serum from a HSV1-infected person that exhibited cross-reactivity with HSV2 proteins.

A serum sample was considered negative if there were no bands on either the HSV1 or the HSV2 strip corresponding to HSV bands on the control strip. HSV1-positive samples were those exhibiting a predominance of binding to the HSV1 strip and a lack of HSV2-specific bands. In samples that also exhibited binding to the HSV2 strip, the pattern was compared with the cross-reactive control, and binding to the HSV2-specific gG-2 band was ruled out before the sample was considered HSV1-positive only. HSV2-positive samples were those that exhibited a predominance of binding to the HSV2 strip along with reactivity to gG-2. If a gG-2 band was not observed, the serum was adsorbed against HSV1 and -2 proteins and the test repeated. HSV1- and HSV2-positive samples showed a full antibody profile on both HSV1 and HSV2 strips, including reactivity to gG-2. Each participant’s HSV serological results were thus categorized according to whether the serum reacted to HSV1 antigens, HSV2 antigens, both, or neither. This method classifies only ~2% of samples (25).

Evidence of previous HPV infection was measured by using ELISA to determine antibody response to the HPV16 L1 capsid protein, a virus-like particle, and by the presence of HPV DNA, as described previously (24).

**Data Analysis.** We created analytic variables to describe each case and control subject’s cigarette smoking status according to his/her status as of reference date (current smoker, former smoker, never smoker) and total pack-years of cigarette smoking. Similarly, we classified each case and control subject’s consumption of alcoholic beverages according to his/her lifetime average number of alcoholic beverages consumed per week.

A participant’s serum was considered HSV1-positive if it reacted to HSV1 antigens, regardless of whether the serum was also HSV2-positive or not, and HSV1-seronegative otherwise. HSV2 seropositivity or seronegativity was categorized similarly. Because some oral herpes infections are caused by HSV2, we investigated whether adjustment for HSV2 serostatus affected the measure of association between HSV1 and OSCC.

We first categorized the HPV genotyping results according to whether or not the tissue contained any oncogenic HPV DNA (HPV types 16, 18, 31, 33, or 35). To increase the power to detect interactions between HPV and HSV1, we considered a case subject to be HPV DNA-positive if he or she had evidence of oncogenic HPV DNA in either his or her tumor tissue or in exfoliated oral tissue. HPV genotyping results from the cases’ tumor tissue samples were included in this classification because surgical removal of tumors may have eradicated all evidence of prior HPV infection in the relevant tissue. The ELISA cutoff used to categorize participants as seropositive for antibody response to HPV16 capsids was calculated as described previously (24).

We used standard methods for the statistical analysis of case-control studies (26). The OR for the association between OSCC risk and factors under study was estimated by using unconditional logistic regression. Because the controls had been frequency-matched to cases on the basis of age categories and sex, analyses were repeated by using conditional logistic regression (27). We used polytomous regression to make comparisons between controls and OSCC patients with tumors at different sites or OSCC patients categorized by stage at diagnosis. CIs (95%) were calculated by using the standard errors from the corresponding logistic regression models and the normal approximation. All reported Ps are two-sided. Unless otherwise stated, the ORs presented are adjusted for age, cigarette smoking (continuous pack-years), alcohol consumption (continuous average number of alcoholic beverages consumed per week during lifetime), sex, and income (≤$15,000, $15,000–$59,000, reference group includes three incomes not made known during the interview), and ≤$60,000. Excluding the three individuals with unreported incomes from the reference group did not affect any of the results. Additional adjustments for race or education also did not affect the results.

The appropriateness of the standard logistic regression models was examined by using the Hosmer-Lemeshow goodness-of-fit test (27). We also evaluated whether the individual coefficients changed to an appreciable degree when any particular individuals were removed from the model. There was no evidence of lack-of-fit or of extremely influential observations.

To determine whether the association between antibody response to HSV1 and OSCC was modified by either cigarette smoking, prior HPV infection, or heavy alcohol consumption, we used the approach described by Rothman (28) to create indicator variables representing the combination of these factors. From logistic regression models including these indicator variables as well as covariates, we calculated the RERI to quantify the joint association of antibody response to HSV1 and either smoking, HPV DNA positivity, HSV16 seropositivity, or heavy alcohol consumption with OSCC risk. The RERI is calculated as the joint exposure OR plus one, minus the sum of the individual exposure ORs. Values of RERI that differ from zero are consistent with statistical interaction on an additive scale. That is, when the RERI exceeds zero the combination of two causal factors in some persons in the population results in the development of more cases of disease than would be predicted based on the sum of the added risks associated with exposure to the individual factors. We computed 95% CIs for RERI by estimating the variances and covariances of the fitted coefficients of the indicator variables and applying delta method-based formulas developed by Hosmer and Lemeshow (29).

**RESULTS**

Case and control participants were both predominantly Caucasian and were otherwise similar with respect to race (Table 1). Case subjects were less likely to have attended college and tended to report lower incomes than control participants. Risk of OSCC was strongly associated with cigarette smoking and alcohol consumption, and the risk of OSCC associated with heavy smoking and heavy drinking combined exceeded that expected in the presence of either exposure alone (Table 1).

Among the 260 OSCC patients studied, 190 (73.4%) tested positive for HSV1 antibodies of whom 57 (22.0% overall) were also HSV2-seropositive. Among the control participants, 287 (64.7%) were HSV1 antibody-positive of whom 59 (13.3% overall) were both HSV1- and HSV2-seropositive. An additional 22 case (8.5%) and 38 (8.6%) control participants tested positive for HSV2 antibodies only. Case subjects were 1.3 times more likely to be HSV1-seropositive than were control subjects, after adjusting for age, sex, cigarette smoking, alcohol consumption history, and income (95% CI, 0.9–2.0; Table 2).

The association between HSV1 antibody positivity and OSCC risk varied by tumor site; the adjusted relative risk of tonsillar tumors (n = 49) associated with HSV1 antibody response was 2.4-fold (95% CI, 1.1–5.4; P = 0.03; Table 3). Patients who had tumors of the gums (OR, 1.8), of other unspecified pharyngeal sites (OR, 1.9), or of the palate (OR, 2.2) were also more likely to be HSV1-seropositive, but
CI, 0.6 increased risk of OSCC among non-cigarette smokers (OR, 1.0; 95% CI, 0.3–5.4) restricted to never-smokers (adjusted OR, 5.7; 95% CI, 2.8–11.9). HSV1 infection was strengthened when the referent group was restricted to ever-smokers (adjusted OR, 1.3; 95% CI, 0.7–2.4) at any other tumor sites.

Prior HSV1 infection was associated with a greater increased risk of OSCC among current cigarette smokers (OR, 4.2; 95% CI, 2.4–7.1) than might be expected due to cigarette smoking alone (OR, 2.3; 95% CI, 1.2–4.2), although seropositivity was not associated with any increased risk of OSCC among non-cigarette smokers (OR, 1.0; 95% CI, 0.6–1.7; RERI, 1.8; 95% CI, 0.0–3.7; Table 4). The association between OSCC and combined exposure to current cigarette smoking and HSV1 infection was strengthened when the referent group was restricted to never-smokers (adjusted OR, 5.7; 95% CI, 2.8–11.9).

Because the finer categorization weakened the ability of the study to explore interactions and to adjust for relevant covariates, we report the results from the dichotomous smoking variable only (Table 4). The association between HSV1 infection and OSCC did not depend either on alcohol consumption history (Table 4) or on sex (data not shown).

Among participants whose tumor tissue or buccal scrapes tested positive for oncogenic HPV DNA, HSV1 antibody-positivity was associated with a 2.0-fold increased risk of OSCC (95% CI, 0.8–4.9), whereas HSV1 seropositivity was associated with only a 1.3-fold increased risk of OSCC among those without oncogenic HPV DNA (95% CI, 0.8–2.1; Table 4). All but one of the oncogenic HPV DNA-positive individuals tested positive for HPV16 DNA. Seropositivity to HSV2 did not increase the risk of OSCC, regardless of whether individuals were positive or negative for high-risk HPV DNA. The interaction between HSV1 and HPV infection was also explored by using HPV serum antibody positivity as a measure of HPV infection. Seropositivity to both HSV1 and HPV16 was associated with a 2.6-fold increased risk of OSCC (95% CI, 1.5–4.4), weaker synergism than that observed when oncogenic HPV DNA was measured directly in oral tumor tissue or buccal scrapes.

The association between HSV1 seropositivity and OSCC incidence did not consistently vary depending on the elapsed time between the diagnosis and interview (data not shown). HSV1 antibody positivity was associated with OSCC to a slightly stronger degree among patients whose cancers had spread regionally or distant to the primary tumor at the time of diagnosis (adj OR, 1.0; 95% CI, 0.4–2.6 for in situ tumors; adjusted OR, 1.2; 95% CI, 0.7–2.0 for localized tumors; and adjusted OR, 1.5; 95% CI, 0.9–2.4 for tumors that had spread). A similar increase in the HSV1-exposure OR (from 1.1 to 2.0 for localized tumors; adjusted OR, 1.2; 95% CI, 0.7–2.1) was observed among patients with cancers whose seropositivity was associated with OSCC to a slightly stronger degree among patients whose cancers had spread regionally or distant to the primary tumor at the time of diagnosis (adjusted OR, 1.0; 95% CI, 0.4–2.6 for in situ tumors; adjusted OR, 1.2; 95% CI, 0.7–2.0 for localized tumors; and adjusted OR, 1.5; 95% CI, 0.9–2.4 for tumors that had spread). A similar increase in the HSV1-exposure OR (from 1.1 to 2.0 for localized tumors; adjusted OR, 1.2; 95% CI, 0.7–2.1) was observed among patients with cancers whose seropositivity was associated with OSCC to a slightly stronger degree among patients whose cancers had spread regionally or distant to the primary tumor at the time of diagnosis (adjusted OR, 1.0; 95% CI, 0.4–2.6 for in situ tumors; adjusted OR, 1.2; 95% CI, 0.7–2.0 for localized tumors; and adjusted OR, 1.5; 95% CI, 0.9–2.4 for tumors that had spread).
Furthermore, the host immune response to HSV1 may itself indirectly enhance cancer development, because cytokines released either during an inflammatory response to infected cells or following HSV1-induced cell death could stimulate the proliferation of neighboring cells.

In our study population, HSV1 seropositivity was significantly associated with OSCC incidence only among individuals who had other OSCC risk factors. This observation supports the hypothesis that HSV1 infection may act as a cofactor in disease progression, perhaps by potentiating the carcinogenicity of other exposures through the mechanisms described above. The fact that HSV1 seropositivity was associated with a particularly increased risk of OSCC of the tonsil, an organ rich in lymphatic tissue, is also consistent with a role for HSV1 as a suppressor of local immune function.

Experimental models offer further insights into the mechanisms through which HSV1 infection may interact with specific exposures, such as tobacco and HPV infection, to contribute indirectly to OSCC risk. For example, HSV1-infected cells are usually lysed in the course of HSV replication, but cell lysis of HSV1-infected oral epithelial cells is inhibited in the presence of tobacco extracts (20). As cells harboring the inactive virus do not lyse, this observation may indicate that tobacco inactivates HSV1. Inactivation of HSV1, in turn, is thought to favor the development of malignancy by prolonging the life of mutagenized cells that would otherwise die during the normal HSV lytic cycle (13, 20). In the absence of such attenuation of the HSV life cycle, mutagenized cells would be less likely to survive long enough to undergo malignant transformation. The observed joint association between cigarette smoking, HSV1, and OSCC risk is compatible with the hypothesis that tobacco-derived chemicals inhibit viral replication and cell lysis, thereby increasing the carcinogenicity of the virus.

HPV infection is independently associated with the risk of OSCC in this and other study populations (24, 33) and laboratory studies show that HPV may also depend on the action of cofactors to transform cells to a malignant phenotype (21). HSV1 could potentiate the HPV-induced transformation of cells, as evidenced by: (a) 4-fold

### DISCUSSION

Although HSV1 has been suspected to play a role in the etiology of OSCC since the 1960s (30, 31), few rigorously designed epidemiological studies have tested this hypothesis. We found that in Washington State, OSCC cases were only slightly more likely to be HSV1-seropositive than healthy age- and sex-matched controls. This increase was limited largely to those individuals who smoked cigarettes or had evidence of HPV infection, among whom HSV1 seropositivity was associated with an ~2-fold increase in the risk of OSCC relative to HSV1-seronegative individuals with similar tobacco or HPV exposure.

It has been postulated that HSV1 might directly transform cells to a malignant phenotype through its mutagenic properties, possibly inducing chromosomal rearrangements, or by amplifying host genes involved in carcinogenesis (7–9, 13, 19–21). Some characteristics that help HSV1 evade the human immune system could also indirectly benefit malignant cells. For example, HSV1 infection has been shown to block apoptosis in infected cultured human epithelial cells (3, 10). Although not in itself sufficient to transform cells to a malignant phenotype, the prevention of programmed cell death could plausibly extend the longevity of cells that have already undergone genetic damage due to other agents (11, 12, 32). HSV1 also appears to inhibit immune function that would otherwise play a potential role in local tumor surveillance, such as natural killer cell-mediated cytotoxicity. After coming into contact with HSV-infected cells, natural killer cells are unable to lyse other targets, and this inhibition is irreversible (4).

Furthermore, the host immune response to HSV1 may itself indirectly enhance cancer development, because cytokines released either during an inflammatory response to infected cells or following HSV1-induced cell death could stimulate the proliferation of neighboring cells.

A 1.5) was noted among cases receiving radiation as compared with those not treated with radiation. Because disease staging is so highly correlated with radiation treatment, it was not possible to examine the association between HSV1 seropositivity and staging at diagnosis independently of treatment (and vice versa).

## Table 4 OR risk in relation to HSV1 antibody response according to cigarette smoking, alcohol consumption, and evidence of prior HPV infection

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>HSV1 antibodies</th>
<th>Cases a (%)</th>
<th>Controls b (%)</th>
<th>OR c</th>
<th>95% CI d</th>
<th>RERI (95% CI e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current cigarette smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not current</td>
<td>No</td>
<td>34 (13)</td>
<td>117 (27)</td>
<td>1.0</td>
<td>Referent</td>
<td>1.8 (0.0-3.7)</td>
</tr>
<tr>
<td>Yes</td>
<td>76 (29)</td>
<td>226 (51)</td>
<td>1.0</td>
<td>0.6-1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>No</td>
<td>36 (14)</td>
<td>39 (9)</td>
<td>2.3</td>
<td>1.2-4.2</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>114 (44)</td>
<td>59 (13)</td>
<td>4.2</td>
<td>2.4-7.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pack-years cigarette smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20 pack-years</td>
<td>No</td>
<td>27 (10)</td>
<td>103 (23)</td>
<td>1.0</td>
<td>Referent</td>
<td>1.8 (~0.1-3.8)</td>
</tr>
<tr>
<td>Yes</td>
<td>56 (22)</td>
<td>197 (45)</td>
<td>1.0</td>
<td>0.6-1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥20 pack-years</td>
<td>No</td>
<td>43 (16)</td>
<td>52 (12)</td>
<td>2.9</td>
<td>1.5-5.4</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>134 (52)</td>
<td>87 (20)</td>
<td>4.7</td>
<td>2.7-8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15 drinks/wk</td>
<td>No</td>
<td>46 (18)</td>
<td>130 (29)</td>
<td>1.0</td>
<td>Referent</td>
<td>0.6 (~0-9 to 2.2)</td>
</tr>
<tr>
<td>Yes</td>
<td>118 (45)</td>
<td>242 (55)</td>
<td>1.3</td>
<td>0.8-2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥15 drinks/wk</td>
<td>No</td>
<td>24 (9)</td>
<td>26 (6)</td>
<td>1.7</td>
<td>0.8-3.5</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>72 (28)</td>
<td>43 (10)</td>
<td>2.6</td>
<td>1.5-4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV16 antibody</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>No</td>
<td>37 (14)</td>
<td>101 (23)</td>
<td>1.0</td>
<td>Referent</td>
<td>0.8 (~0.3-2.0)</td>
</tr>
<tr>
<td>Yes</td>
<td>89 (34)</td>
<td>184 (42)</td>
<td>1.1</td>
<td>0.7-1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>No</td>
<td>33 (13)</td>
<td>55 (12)</td>
<td>1.6</td>
<td>0.9-3.1</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>100 (39)</td>
<td>101 (23)</td>
<td>2.6</td>
<td>1.5-4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oncogenic HPV DNA in tumor or scrape</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>No</td>
<td>53 (22)</td>
<td>129 (33)</td>
<td>1.0</td>
<td>Referent</td>
<td>3.3 (~5.2 to 12)</td>
</tr>
<tr>
<td>Yes</td>
<td>144 (58)</td>
<td>244 (62)</td>
<td>1.3</td>
<td>0.8-2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>No</td>
<td>13 (5)</td>
<td>7 (2)</td>
<td>5.4</td>
<td>1.9-16</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>36 (15)</td>
<td>11 (3)</td>
<td>9.0</td>
<td>4.0-20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Excludes six control subjects for whom pack-years of cigarette smoking and income could not be determined.

All ORs are based on interviewed case subjects and control subjects from whom blood specimens were obtained and tested and are adjusted for age (continuous), sex, and income indicators (~$15,000, $15,000–$39,000 or unreported (reference group), or ~$60,000). ORs for joint association of HSV1 antibody response and current smoking or pack-years of smoking are also adjusted for average number of alcoholic beverages per week (continuous). ORs for joint association of HSV1 antibody response and alcohol consumption are also adjusted for pack-years of smoking (continuous). Odds ratios for joint association of HSV1 antibody response and HPV DNA status are also adjusted for pack-years of smoking (continuous) and average number of alcoholic beverages per week (continuous).

Reference group for OR calculation.

Average alcohol consumption over lifetime.
increases in the amount of HPV18 DNA in HSV1-infected as compared with mock-infected human cells (21); (b) the ability of HSV1 genes to induce the activation of HPV18 gene expression in both cervical cancer and squamous cell carcinoma epithelial cell lines (22); and (c) increased amplification of HPV DNA sequence copy numbers as well as increased integration of HPV DNA into cells in the setting of HSV1 infection (23). We found that HSV1 seropositivity was associated with a 2-fold increase in risk among individuals harboring oncogenic HPV DNA in either tumor tissue samples or oral tissue scrapes, whereas HSV1 seropositivity was not associated with an increase in risk among HPV DNA-negative participants. This difference is consistent with experimental results, but it is also consistent with stratum-to-stratum random variation as reflected by the width of the 95% CI and its inclusion of zero.

Several aspects of our study limit the strength of our conclusions. First, only ~60% of eligible cases and 60% of eligible controls participated in the study. Deaths prior to recruitment comprised the largest proportion of nonparticipating cases (15.4% of total eligible cases), whereas refusal to be interviewed was the most common reason for the low participation rate among controls (32.1% of total eligible controls). Inasmuch as the nonparticipants differ from those who participated in the study with respect to HSV1 seropositivity, and such variation itself varied by case-control status, the results of our study may be biased. We have no reason to suspect that a history of HSV1 infection negatively affects survival of cancer patients; such a relationship could cause the cases who survived to interview to underrepresent the prevalence of HSV1 seropositivity among OSCC patients overall, potentially leading to an underestimate of the true association between HSV1 antibodies and OSCC risk.

Conversely, some recent studies suggest that HSV infection is associated with favorable survival among OSCC patients (34). To the extent that HSV1 infection is directly correlated with HSV1 seropositivity, failure to enroll some cases because of early death could have resulted in spurious associations between OSCC risk and HSV1 seropositivity, either alone or in conjunction with other characteristics. HSV1 seropositivity was the same, however, regardless of HPV DNA status, either among controls, among cases, or among the whole population (data not shown). Finally, we noted no relationship between HSV1 seroprevalence and the time elapsed between diagnosis and interview among the cancer patients studied (median, 8.4 and 8.2 months among HSV1-seronegative and -seropositive case participants, respectively; Mann-Whitney, $P = 0.71$).

Second, we measured circulating levels of anti-HSV1 antibodies as a marker of oral HSV1 infection. Whereas HSV1 infections mainly occur in the oral mucosa, and HSV2 infections mainly occur on the genitalia (1, 5, 35), up to 18% of HSV1 seropositivity (36) may have arisen through non-oral infections and some HSV2 seropositivity through oral infections. Our inability to measure oral HSV1 and HSV2 infections directly may have diluted our measures of association in this study. Exclusion of the 22 individuals who had a prior history of clinically diagnosed genital herpes infections did not materially change our results regarding HSV1 seropositivity, either overall or in combination with HPV infection or cigarette smoking (data not shown). Thus, genitally acquired HSV1 is unlikely to have been positive for the positive associations seen in our study. Furthermore, although HSV2 might be postulated to have the same carcinogenic potential as HSV1, its relative absence in the oral cavity weakens such a hypothesis. Adjustment for HSV2 serostatus did not affect the results of any of our analyses, nor was HSV2 seropositivity associated with OSCC risk either overall or in combination with HPV infection or cigarette smoking (data not shown).

Third, blood samples obtained from case participants after treatment may not accurately reflect HSV1 seropositivity prior to diagnosis. Radiotherapy and oral surgery are known to reactivate latent herpes simplex infections (5). If such reactivations cause a concomitant rise in HSV1 antibody titers, seropositive case participants may have been less likely than seropositive control participants to be falsely classified as seronegative, potentially leading to spurious associations. If the cancer therapy indeed caused such differential misclassification, we would have expected HSV1-seropositive cases to have been more likely than HSV1-seronegative cases to have had blood samples taken closer to the time of diagnosis. The amount of time since cancer diagnosis was the same, however, regardless of HSV1 antibody status (see above).

Fourth, data that we obtained through interviews are also subject to misclassification. In particular, it is plausible that participants may have underreported their use of tobacco and alcohol, the two most important risk factors for OSCC in developed nations. The magnitude of the associations we observed between these exposures and OSCC, however, are comparable with those reported by others (37, 38). To the extent that the dichotomous alcohol or tobacco exposures were misclassified, the study may have reduced ability to detect any underlying interaction between HSV1 seropositivity and either of the exposures.

Fifth, confounding of the results by unmeasured covariates cannot be ruled out. For example, the conjuction of OSCC and HSV1 infection in some individuals may reflect an unknown immunological characteristic that confers susceptibility to both diseases rather than a causal relationship between the two. The fact that such associations were mainly observed in the presence of HPV infection or cigarette smoking detracts from the plausibility of such a scenario.

Sixth, although this is the largest epidemiological study addressing the relationship between HSV1 and OSCC risk, it was still relatively underpowered to test hypotheses regarding the interaction of HSV1 infection with other OSCC risk factors. Our findings on the association between HSV1 infection and OSCC do not entirely agree with those reported by others. Because we based our results on a dichotomous antibody classification, they are not directly comparable with the observations reported by Shillitoe et al. (14, 15) that HSV1 antibody titers were positively related to oral cancer. Their data suggest, however, that antibody titers did not differ appreciably between clinic controls who smoked and oral cancer cases. The exclusion from these studies of participants with a history of recurrent herpetic infection also complicates any comparison between their results and those we present here.

We previously found no overall association between HSV1 seropositivity and the incidence of OSCC (17). As that study was relatively small (131 cases and 136 controls), it may have been underpowered to identify main effects with such a common exposure, and at that time we did not attempt to identify whether the association between HSV1 and OSCC depended on smoking history. In contrast, a recent case-control study (56 cases and 56 controls) conducted in Pakistan demonstrated a 10-fold association between HSV1 seropositivity and OSCC after controlling for age, sex, and tobacco use (39). Although the analysis chosen by the authors did not allow for adjustment for other covariates, the strength of association they observed suggests it is not entirely due to uncontrolled confounding. Only 2 of the 56 oral carcinoma cases they examined, however, tested negative for HSV1 antibodies. Misclassification of even a small number of HSV1-seropositive cases could therefore have greatly exaggerated their estimate of the association between HSV1 and OSCC. It is also possible that HSV1 plays a more prominent role in OSCC etiology in settings where the prevalence of other strong OSCC risk factors differs from that in the U.S. (37). The Pakistani general population, for example, experiences higher prevalences of both heavy smoking and the chewing of betel quid.
Similarly, in a large (354 cases and 354 controls), population-based case-control study of OSCC in Sweden, Schildt et al. (40) found that self-reporting of clinically apparent herpes infection was associated with a nonsignificant increase in risk (OR: 1.9; 95% CI: 0.7–4.5). Three major differences prevent a direct comparison between these results and those of our study. First, these analyses were not adjusted for potential confounding factors, and in particular, cigarette smokers are more likely to be HSV1-seropositive than tobacco nonusers (39). Second, HSV1 exposure in the Swedish study was measured through mailed questionnaires as opposed to measuring antibody reactivity. Third, approximately half of the Swedish OSCC patients with histories of oral infections had been diagnosed with lip cancer. We did not include lip cancers in our study because these tumors, in contrast to other OSCC, are strongly related to heavy sun exposure (37, 38). Because sun exposure can lead to both oral herpetic reactivation and an increase in lip cancer risk, inclusion of lip cancer cases in the Swedish study may have inflated estimates of the association between the two.

**Conclusion.** We found that HSV1 was particularly associated with OSCC risk when other risk factors, such as cigarette smoking or a history of HPV infection, are present. Because of the high prevalence of HSV1 infection (from 60–80% in every country studied; Refs. 1 and 35), even if HSV1 infection is only associated with OSCC among smokers it could contribute to a substantial portion of the disease among smokers. This effect modification is well supported by experimental data; future research should address whether it is also supported by evidence from other populations.

**ACKNOWLEDGMENTS**

This work was made possible through the collaboration and efforts of many individuals: Elizabethickman, who managed data and specimen collection activities; the staff of the Cancer Surveillance System, who performed case subject ascertainment; Dan Edelson, Marion Knudson, Scott McIntosh, Barbara Hansen, Laura Callahan, and Aimee Lowe, who recruited and interviewed case and control subjects; Kay Byron, Dick Jacke, Raymond Miller, Kenneth Scholes, and Judith Kuksin, who provided computer support; Jean Jue, who performed data entry; David Doody, who performed data cleaning and management; Joseph Carter and Gregory Wipf, who conducted HPV antibody response assays; and Er-Jia Mao and Shixuan Huang, who conducted HPV genotyping assays.

**REFERENCES**

Serologic Evidence of Herpes Simplex Virus 1 Infection and Oropharyngeal Cancer Risk
