# Cervical and Vulvar Cancer Risk in Relation to the Joint Effects of Cigarette Smoking and Genetic Variation in Interleukin 2

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## Abstract

**Cigarette smoking is an established cofactor to human papillomavirus (HPV) in the development of cervical and vulvar squamous cell carcinoma (SCC), and may influence risk through an immunosuppressive pathway. Genetic variation in *interleukin 2 (IL2)*, associated in some studies with the inhibition of HPV-targeted immunity, may modify the effect of smoking on the risk of HPV-related anogenital cancers. We conducted a population-based case-only study to measure the departure from a multiplicative joint effect of cigarette smoking and *IL2* variation on cervical and vulvar SCC. Genotyping of the four *IL2* tagSNPs (rs2069762, rs2069763, rs2069777, and rs2069778) was done in 399 cervical and 486 vulvar SCC cases who had been interviewed regarding their smoking history. Compared with cases carrying the rs2069762 TT genotype, we observed significant departures from multiplicativity for smoking and carriership of the TG or GG genotypes in vulvar SCC risk [interaction odds ratio (IOR), 1.67; 95% confidence interval (CI), 1.16-2.41]. Carriership of one of three diplotypes, together with cigarette smoking, was associated with either a supramultiplicative (TGCT/GGCC; IOR, 2.09; 95% CI, 0.98-4.46) or submultiplicative (TTCC/TGTC; IOR, 0.37; 95% CI, 0.16-0.85 or TGCT/TGCC; IOR, 0.37; 95% CI, 0.15-0.87) joint effect in vulvar cancer risk. For cervical SCC, departure from multiplicativity was observed for smokers homozygous for the rs2069763 variant allele (TT versus GG or GT genotypes; IOR, 1.87; 95% CI, 1.00-3.48), and for carriership of the TTCC/TTCC diplotype (IOR, 2.08; 95% CI, 1.01-4.30). These results suggest that cervical and vulvar SCC risk among cigarette smokers is modified by genetic variation in *IL2*.** (Cancer Epidemiol Biomarkers Prev 2008;17(7):1790–9)

## Introduction

Persistent oncogenic human papillomavirus (HPV) infection is etiologically linked to all cervical cancers and a large subset of vulvar cancers (1). The HPV-dependent vulvar cancers are associated with nonkeratinizing basaloid or warty vulvar intraepithelial neoplasia, and primarily affect younger women. They bear a remarkable resemblance to cervical squamous intraepithelial neoplasia and cancer, and are associated with similar HPV types and cofactors (2, 3).

Cigarette smoking is among the most well-established HPV cofactors in the etiology of these malignancies (4). Current smokers are at ~2-fold to 3-fold increased cervical squamous cell carcinoma (SCC) risk (5), and at >3-fold increased vulvar SCC risk (2, 3), whereas former cigarette smokers tend to be at little or no increased risk (5, 6). Studies have also observed a positive association between cervical SCC risk and increased duration of smoking (5, 7), although this trend seems to be driven by the high proportion of long-term smokers who are also current smokers (7). Experimental evidence linking smoking cessation and a decrease in cervical lesion size (8) also highlights the important role of current cigarette smoking in cervical SCC risk.

The biological mechanism whereby cigarette smoking increases cervical and vulvar SCC risk remains largely undetermined (9). One possibility is that smoking enhances immunosuppression (8). The importance of the adaptive immune response in HPV-associated cancer risk is emphasized by studies showing that HIV-infected women have a substantially increased risk of developing cervical and vulvar cancer (10, 11), and women with drug-induced immunosuppression are 9 times more likely than the general population to develop an HPV infection, and 16 times more likely to develop cervical cancer (12). In immunocompetent patients capable of preventing persistent HPV infection and related neoplastic changes, Th1 cytokines such as interleukin 2 (IL2)

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propagate a T lymphocyte–mediated immune response to HPV and tumor antigens (13-16). IL2 is a T lymphocyte–derived cytokine that is secreted minutes after the activation of a T lymphocyte receptor by an antigen bound to a MHC receptor on an antigen-presenting cell. IL2 acts in an autocrine manner by binding the IL2 receptor on activated T lymphocytes and inducing the transcription of other Th1 cytokines, which together, propagate the T lymphocyte response (17). IL2 is considered to be a key component of the adaptive immune response to HPV infection and the development and growth of tumors driven by the viral oncogenes (18, 19).

Experimental studies show an influence of both cigarette smoking (20-24) and genetic variation (25) on IL2 expression, suggesting the possibility that cigarette smoking and inherited genetic variation in *IL2* interact to increase cervical and vulvar SCC risk. We conducted the present study to test that hypothesis.

## Materials and Methods

**Study Design.** Assessing the joint effect of cigarette smoking and *IL2* nucleotide variation on HPV-dependent cancers would ideally involve assessing the interaction effect among women who have persistent oncogenic HPV infection (26). Practically, however, oncogenic HPV infection in the general population of adult women identified with current detection methods is uncommon (between 2% and 12%), and persistent infection is rare (27). A case-only design avoids the difficult task of selecting a control group with persistent HPV infection. Under the assumption of independence between cigarette smoking and variation in *IL2,* the interaction odds ratio (IOR) from a case-only design provides an estimate of effect modification equivalent to that derived from a case-control study under a multiplicative model (28). In addition, the case-only design offers higher precision to estimate the IOR compared with a standard case-control design (29).

**Study Population.** This study was ancillary to a large, population-based case-control study focused on host and environmental factors that contribute to HPV-related anogenital cancer risk (2, 30). Briefly, the case-control study attempted to recruit all 18- to 74-year-old residents of King, Pierce, and Snohomish counties, Washington, diagnosed with incident invasive cervical and invasive or *in situ* vulvar cancer between January 1986 and June 1998 or between January 2000 and December 2004. Cases were ascertained through the Cancer Surveillance System, a population-based registry that is a part of the National Cancer Institute's Surveillance, Epidemiology, and End Results program (31). To help ensure comparability between the cases and controls, who were identified and recruited using a one-step modification of the Waksberg-Mitofsky method of random-digit telephone dialing (32, 33) and frequency-matched to cases by 5-year age groups, only cases with residential telephones were eligible for the study. Cases with tumors that were not SCC (e.g., adenocarcinoma) were excluded from this ancillary study as those histologies are not related to cigarette smoking. Non–Caucasian women were excluded from this study because they comprised <10% of the original study population, precluding meaningful subgroup analyses stratified by race whereas increasing the possibility of bias due to population stratification. A sample of Caucasian controls from the parent study was included in this ''case-only'' study to test the assumption of independence between genotypes of *IL2* polymorphism and cigarette smoking. The cervical cancer control group was restricted to women without prior hysterectomy, thus reflecting the population from which the cases arose. No such restrictions were placed on the vulvar cancer controls.

**Data and Specimen Collection.** In the case-control study, in-person interviews were conducted to elicit information on demographic and other characteristics with a known or suspected relationship to anogenital cancer, including cigarette smoking. A woman was considered a smoker if she reported smoking 100 or more cigarettes in her lifetime. Venous blood samples were drawn at the time of the interview to provide serum samples for HPV16 and HPV18 antibody testing as described previously (34). Beginning in 1991, 5 years after the start of the study, we expanded the blood collection to include samples from which DNA could be isolated. We also re-contacted the cervical, but not the vulvar, cancer cases interviewed in the earliest years of the study and asked them to provide these additional blood samples. A small proportion of study participants (3%) preferred to donate a buccal cell sample, which was collected using a standardized oral rinse procedure, in place of blood. We attempted to retrieve archival tissue blocks from biopsy or surgery to determine the presence and type of HPV DNA in the tumors of the cervical and vulvar cancer cases. HPV DNA typing on tumor tissue was done using PCR methods, as described in detail previously (35).

**Response Rates.** Among the 1,189 eligible cervical SCC patients identified for the parent case-control study, 744 (62.6%) were interviewed, and among those interviewed, 674 (90.6%) provided a specimen from which DNA could be obtained. A similar proportion of vulvar SCC cases were interviewed (807 of the 1,194; 67.6%); however, specimens from which DNA could be obtained were only collected from 73.4% of participating vulvar cancer cases. This percentage is largely affected by the fact that, as described above, the early version of the parent study protocol did not include the collection of blood specimens from which DNA could be isolated, and that the vulvar cancer cases, unlike the cervical cancer cases, were not re-approached for these specimens once the protocol was changed. Reasons for nonparticipation were largely similar for the two cancers and included doctor refusal to allow us to contact the patient (5% and 6%, for cervical and vulvar cases, respectively), refusal of the patient to participate or our inability to locate the patient (22% and 24%), or patient death (10% and 3%). Drawn from the Caucasian participants who had a sufficient DNA sample at the time of this study, our analyses included 399 cervical and 490 (434 *in situ*) vulvar SCC cases. Four vulvar SCC cases (3 *in situ*) were not included in any of the tables because a genotyping result could not be obtained from their samples for any of the polymorphisms included in this study, resulting in a total of 486 vulvar SCC cases. Sixty-three percent ($n$ = 251) of cervical cancer cases and 71% ($n$ = 347) of

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vulvar cancer cases included in this study had tumor tissue available that had been tested for HPV DNA. Sixty-seven percent of eligible control women agreed to participate, and 83.9% ($n$ = 1,372) of those interviewed donated a blood sample from which DNA could be obtained.

The parent population-based study had no measure of HPV DNA in the cervix or vulva for control subjects. Yet, the assessment of independence of cigarette smoking and *IL2* genotypes is best in a control sample that comes from the same pool of HPV infected women that give rise to the cases in this study. Thus, among the 1,094 eligible controls with genomic DNA available, we included in the present study only those that were positive for HPV16 or HPV18 L1 serum antibodies, a measure of past exposure to the virus, by a virus-like particle assay ($n$ = 236; ref. 34).

**TagSNP Selection.** Information on *IL2* nucleotide variation was obtained from the SeattleSNPs Variation Discovery Resource (36).[6] Briefly, SeattleSNP has resequenced exons, introns, and 1,000 bp or more on the 5′ and 3′ ends of each target gene in DNA from 23 Centre d'Etude du Polymorphisme Humain parents of European descent and 24 African-Americans, obtained from the Coriell Repository (Camden, NJ). Using the European descent data, all single-nucleotide polymorphisms (SNPs) with a variant allele frequency of at least 5% were identified; 7 out of the 10 SNPs met this criterion. Next, a pairwise $r^2$ cutpoint of 0.80 was used to delineate groups of highly correlated SNPs (37) and one polymorphism (i.e., tagSNP) per group was selected to be genotyped. When more than one possible tagSNP for a particular group of correlated SNPs was identified, information regarding putative function reported in the literature and location of the SNP informed tagSNP selection. The National Center for Bioinformatics dbSNP build 127 reference sequence number for the four selected *IL2* tagSNPs are rs2069762, rs2069763, rs2069777, and rs2069778.

**Genotyping of *IL2* tagSNPs.** Genomic DNA was extracted from buffy coat aliquots from blood samples, or cell pellets from buccal samples, using a phenol chloroform method (38). Genotyping was done using Pre-Designed or Custom TaqMan genotyping assays from Applied Biosystems following the manufacturer's protocol (Applied Biosystems). Briefly, the assays were conducted in a 5-μL volume containing 5 to 50 ng of genomic DNA, 2.5 μL of the 2× Universal Master Mix with uracil-DNA glucosylase, 200 nmol/L of each assay-specific primer, and 900 nmol/L of each assay-specific FAM and VIC fluorescently labeled probe. Reactions were amplified using a 9700 PCR machine or a 7500 Real-time PCR system (Applied Biosystems) for 50°C for 2 min, 95°C 10 min followed by 40 to 50 cycles of 92°C for 15 to 30 s and 58°C to 60°C for 1 to 1.5 min. The fluorescence release was measured by the 7500 Real-time PCR system using the allelic discrimination setting of the Sequence Detection Software version 1.2.3 (Applied Biosystems). Probe and primer sequences are listed in Supplementary Table S1. Two to three positive controls (samples known to be heterozygous or homozygous for each allele based on sequencing) and negative controls (wells containing no DNA) were included in each reaction plate. Specimens were organized so that the replicate quality control DNA aliquots, which comprised ~10% of the specimens, were distributed throughout the reaction plates. Analysis of these replicates revealed a low discordance proportion of 1%. Laboratory personnel were blinded to all research information about the samples, including the identities of the quality control replicate aliquots.

**Data Analysis.** TagSNP genotypes were tested for consistency with Hardy-Weinberg equilibrium within the HPV-seropositive control sample using a Pearson's $\chi^2$ $P$ value cutpoint of 0.05. The control sample was also used to test for the independence of smoking status and *IL2* tagSNP genotypes. One approach to test for independence is to use logistic regression to model smoking as a dependent variable and genotype as an independent variable among the controls. Alternatively, Umbach and Weinberg (39) proposed a method which offers higher precision that uses a likelihood ratio test (LRT) to compare two nested log-linear models for each tagSNP. In the full model, the logarithm of the expected cell count was the dependent variable that fully parameterizes the joint effect of cigarette smoking and tagSNP genotypes separately for cases and controls. The reduced model fixed the joint effect parameter for the controls at zero. Thus, the LRT comparing these two models is a test of the association between tagSNP genotypes and cigarette smoking among controls. A LRT of $P \leq 0.05$, or an exponentiated joint effect parameter for cigarette smoking and tagSNP genotype among controls [the odds ratios (OR) from the full model] departing substantially from the null, was taken as evidence of a statistically significant lack of independence between cigarette smoking status and *IL2* genotypes. For the cervical cancer analyses, these models were fit after excluding 56 controls without intact uteri, resulting in 180 controls.

For tagSNPs that met the independence criteria, IORs and 95% confidence intervals (CI) were calculated using logistic regression. Separately for the cervical and vulvar cancer case groups, current cigarette smokers were compared with former or never smokers as the outcome variable, and tagSNP genotypes comprised the predictor variables. The IORs represent the departure of the joint effect of *IL2* tagSNP genotypes and current cigarette smoking, from that expected under a multiplicative model, on cervical and vulvar cancer risk. Genotype IORs were calculated without restricting to a particular genetic model, and additional IORs were calculated assuming dominant and recessive penetrance. Genotype IORs were also calculated on the subgroup of vulvar cancer cases testing positive for oncogenic HPV DNA in their tumors or positive for HPV16 or HPV18 L1 serum antibodies ($n$ = 325). Age at diagnosis, tumor stage, education, number of lifetime sexual partners, parity, oral contraceptive use, and family history of anogenital cancer were considered as potential confounding factors of the IORs, but did not have substantial influences and were not included in the final models.

[6] http://pga.gs.washington.edu/data/il2/

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PHASE version 2.1 software (40) was used to statistically infer haplotypes in *IL2*. A log-additive genetic model was assumed to obtain haplotype IORs and 95% CIs using logistic regression. We accounted for some of the uncertainty inherent in statistical determination of haplotypes by including all PHASE-inferred haplotypes into our logistic regression models as separate observations, weighted in proportion to their PHASE-inferred probabilities of being the true haplotype (41). We also calculated IORs and 95% CIs for pairs of haplotypes (diplotypes) using similar weighted logistic regression models. In the sections that follow, SNP alleles in each haplotype are listed from 5′ to 3′ (rs2069762, rs2069763, rs2069777, rs2069778), and the variant allele at each locus is underlined.

The main effect of each tagSNP on cervical and vulvar cancer risk was assessed. Cervical cancer cases and vulvar cancer cases were compared with HPV16 or HPV18 L1–seropositive controls, and subanalyses were conducted in which oncogenic HPV DNA–positive or HPV16- or HPV18 L1–seropositive vulvar cancer cases were compared with HPV16- or HPV18 L1–seropositive controls. Cervical cancer analyses were conducted after excluding controls without intact uteri. Separate logistic regression models were used to estimate genotype-specific ORs and 95% CI for each tagSNP and cancer site.

## Results

Selected characteristics of the cervical and vulvar cancer cases included in this study are presented in Table 1. Eighty-nine percent of the vulvar cancer cases in this study were diagnosed with *in situ* tumors, and 83% of the cervical cancer cases were diagnosed with an invasive tumor staged 2b or less (International Federation of Gynecology and Obstetrics). On average, the vulvar and cervical cancer case groups were similar with respect to HPV positivity, education level, and oral contraceptive use. However, the vulvar cancer cases were older, more likely to be current smokers, had more sexual partners, had fewer live births, and were more likely to have had a family history of anogenital cancer compared with cervical cancer cases.

TagSNP variant allele frequencies ranged from 0.07 to 0.38 (Table 2). We did not find statistical evidence of lack of fit to Hardy-Weinberg equilibrium for any of the tagSNPs. We observed the independence of tagSNP genotypes and cigarette smoking among both cervical and vulvar HPV-seropositive control groups, as indicated by ORs close to the null value and LRT values of $P \geq 0.05$ (Table 2).

Compared with homozygous carriers of the common allele of tagSNP rs2069762 (TT genotype), positive departures from multiplicativity were observed for vulvar cancer cases carrying one (IOR, 1.69; 95% CI, 1.15-2.47), or two (IOR, 1.59; 95% CI, 0.76-3.32) copies of the variant G allele (Table 3). The dominant genetic model showed a similar departure for smokers carrying either the TG or GG genotypes, versus carriers of the TT genotype (IOR, 1.67; 95% CI, 1.16-2.41). A similar departure from multiplicativity was observed when the analysis was restricted to the oncogenic HPV DNA–positive or HPV16- or HPV18 L1–seropositive vulvar cancer cases, TG or GG genotypes versus TT (IOR, 1.92; 95% CI, 1.21-3.04). However, a slightly increased IOR was observed for all women who were tested for either tumor HPV DNA or HPV serology ($n = 363$; IOR, 1.83; 95% CI, 1.20-2.79) compared with women who did not have tumor tissue available for testing ($n = 123$; IOR, 1.25; 95% CI, 0.59-2.67).

In the recessive genetic model, homozygosity for the variant allele of rs2069763 (TT genotype) and cigarette smoking was associated with a significant positive departure from multiplicativity in cervical cancer risk (IOR, 1.87; 95% CI, 1.00-3.48), which was not observed for vulvar cancer (IOR, 0.99; 95% CI, 0.50-1.94). Genotypes of rs2069777 and rs2069778 did not show elevated or reduced IORs with cigarette smoking in either cervical or vulvar cancer risk.

We observed five haplotypes in *IL2*, each uniquely tagged by the presence of a single variant allele, TTCC,

**Table 1. Selected characteristics of cervical and vulvar SCC cases**

| | Cervical cancer cases ($n = 399$) | Vulvar cancer cases ($n = 486$) |
|---|---|---|
| Mean age at diagnosis (y) | 43.1 | 47.4 |
| Tumor stage at diagnosis by FIGO staging (%) | | |
| Vulvar | | |
| 0 | | 88.6 |
| 1+ | | 11.4 |
| Cervix | | |
| <2b | 82.9 | |
| ≥2b | 17.1 | |
| HPV DNA testing (%) | | |
| Not tested | 36.6 | 28.6 |
| Tested | 63.4 | 71.4 |
| Positive result (high-risk types)* | 83.4 | 82.3 |
| Negative result (high-risk types)* | 12.5 | 10.0 |
| Undetermined* | 4.1 | 7.7 |
| Education (%) | | |
| High school or less | 37.9 | 37.9 |
| Less than 4 years of college or technical school | 41.3 | 42.1 |
| 4 years of college or more | 20.8 | 20.0 |
| Cigarette smoking (%) | | |
| Never | 38.4 | 20.2 |
| Former | 26.9 | 22.6 |
| Current | 34.7 | 57.2 |
| Number of lifetime sexual partners (%) | | |
| 1 | 9.6 | 7.6 |
| 2 to 4 | 30.5 | 21.5 |
| 5 to 14 | 44.1 | 43.8 |
| ≥15 | 15.9 | 27.1 |
| No. of births (%) | | |
| 0 | 18.1 | 29.6 |
| 1 | 16.3 | 19.3 |
| 2 | 30.2 | 25.5 |
| ≥3 | 35.4 | 25.5 |
| Duration of oral contraceptive use (%) | | |
| Never or less than 6 mo | 31.4 | 29.6 |
| 6 to 59 mo | 31.4 | 32.3 |
| ≥5 y | 37.2 | 38.1 |
| First degree relative with anogenital cancer (%) | | |
| Yes | 3.7 | 8.0 |
| No | 96.3 | 92.0 |

Abbreviation: FIGO, International Federation of Gynecology and Obstetrics.
*Represents the percentage of tested individuals.

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**Table 2. *IL2* tagSNP characteristics, smoking prevalence, and results from tests of independence of *IL2* tagSNPs and cigarette smoking in controls**

| TagSNP* | Location † | Gene feature ‡ | Alleles (common/variant) | Variant allele frequency | Smoking prevalence§ | Cervix controls | | Vulvar controls | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | OR (95% CI)‖ | *P*¶ | OR (95% CI) | *P* |
| rs2069762 | 495 | 5′ Flanking | T/G | 0.23 | 23% | 1.19 (0.69-2.06) | 0.68 | 1.30 (0.82-2.04) | 0.81 |
| rs2069763 | 993 | Exon 1 | G/T | 0.38 | 23% | 1.11 (0.66-1.85) | 0.48 | 1.08 (0.69-1.67) | 0.85 |
| rs2069777 | 2038 | Intron 1 | C/T | 0.07 | 21% | 1.23 (0.45-3.37) | 0.69 | 0.90 (0.34-2.33) | 0.82 |
| rs2069778 | 2340 | Intron 1 | C/T | 0.18 | 18% | 0.72 (0.35-1.46) | 0.54 | 0.60 (0.31-1.11) | 0.18 |

*rs number refers to the National Center for Bioinformatics dbSNP build 127 reference sequence number.
†Locations are with respect to the first nucleotide position in the National Center for Bioinformatics GenBank entry: accession number AF359939.
‡Location of tagSNP within gene: 5′ flanking is upstream of the first exon of the gene, exon is in the coding region of the gene, intron is between coding regions of the gene.
§Prevalence of current cigarette smoking among all controls ($n$ = 236) who carried at least one copy of the variant allele for each tagSNP.
‖The ORs are the exponentiated joint effect parameters for cigarette smoking and tagSNP genotype among controls from the full model (39) assuming a log-additive genetic model. These can be interpreted as the association between IL-2 tagSNP genotypes and cigarette smoking among controls.
¶LRT *P* value (39).

GGCC, TGCT, and TGTC, or no variant alleles, TGCC (as indicated by the underlined allele; Table 4). Compared with carriers of the most common haplotype, TTCC, cigarette smoking and carriership of any other haplotype did not result in significant departures from multiplicativity in either cervical or vulvar cancer risk. The GGCC haplotype, defined by the variant allele of rs2069762, was associated with a positive, but not statistically significant, departure from multiplicativity in vulvar cancer risk (IOR, 1.34; 95% CI, 0.94-1.92). Compared with carriers of the most common diplotype (TTCC/GGCC), carriership of the TTCC/TTCC diplotype, defined by two copies of the variant allele of rs2069763, and cigarette smoking, together resulted in a positive departure from multiplicative joint effects on cervical (IOR, 2.08; 95% CI, 1.01-4.30), but not vulvar (IOR, 0.85; 95% CI, 0.41-1.78), cancer risk (Table 5). Interestingly, TGCT/GGCC, was associated with a marginally significant positive departure from the multiplicative model in vulvar (IOR, 2.09; 95% CI, 0.98-4.46) but not cervical (IOR, 1.09; 95% CI, 0.47-2.53), cancer risk. Two rare diplotypes were associated with submultiplicative joint effects in vulvar cancer risk, TTCC/TGTC (IOR, 0.37; 95% CI, 0.16-0.85), and TGCT/TGCC (IOR, 0.37; 95% CI, 0.15-0.87).

The ORs for the main effect of each tagSNP on cervical and vulvar cancer risk are presented in Table 6. Compared with the rs2069762 TT genotype, the TG genotype was associated with a marginally significant increased risk of vulvar cancer (OR, 1.28; 95% CI, 0.92-1.78), which was slightly more pronounced when the analysis was restricted to HPV-positive vulvar cancer

**Table 3. IORs between *IL2* genotypes and cigarette smoking on cervical and vulvar cancer risk**

| TagSNP* | Genotype | Cervical cancer cases | | | Vulvar cancer cases | | |
|---|---|---|---|---|---|---|---|
| | | Genotype frequency | | IOR (95% CI) | Genotype frequency | | IOR (95% CI) |
| | | Smokers | Nonsmokers | | Smokers | Nonsmokers | |
| rs2069762 | TT | 0.54 | 0.51 | 1.00 | 0.42 | 0.56 | 1.00 |
| | TG | 0.37 | 0.42 | 0.85 (0.55-1.32) | 0.50 | 0.38 | 1.69 (1.15-2.47) |
| | GG | 0.09 | 0.07 | 1.29 (0.60-2.80) | 0.08 | 0.06 | 1.59 (0.76-3.32) |
| | TG or GG vs. TT † | | | 0.91 (0.60-1.38) | | | 1.67 (1.16-2.41) |
| | GG vs. TT or TG ‡ | | | 1.39 (0.66-2.93) | | | 1.24 (0.61-2.54) |
| rs2069763 | GG | 0.44 | 0.42 | 1.00 | 0.44 | 0.43 | 1.00 |
| | GT | 0.39 | 0.48 | 0.79 (0.50-1.25) | 0.48 | 0.49 | 0.98 (0.67-1.44) |
| | TT | 0.17 | 0.10 | 1.66 (0.86-3.22) | 0.08 | 0.08 | 0.98 (0.48-1.97) |
| | GT or TT vs. GG † | | | 0.94 (0.61-1.43) | | | 0.98 (0.68-1.42) |
| | TT vs. GG or GT ‡ | | | 1.87 (1.00-3.48) | | | 0.99 (0.50-1.94) |
| rs2069777 | CC | 0.86 | 0.85 | 1.00 | 0.86 | 0.80 | 1.00 |
| | CT | 0.14 | 0.15 | 0.96 (0.53-1.74) | 0.14 | 0.19 | 0.65 (0.39-1.06) |
| | TT | | | – | 0.01 | 0.01 | 0.72 (0.04-11.6) |
| | CT or TT vs. CC † | | | – | | | 0.65 (0.40-1.06) |
| | TT vs. CC or CT ‡ | | | – | | | 0.77 (0.05-12.40) |
| rs2069778 | CC | 0.74 | 0.68 | 1.00 | 0.66 | 0.69 | 1.00 |
| | CT | 0.22 | 0.28 | 0.70 (0.43-1.16) | 0.31 | 0.29 | 1.17 (0.78-1.75) |
| | TT | 0.04 | 0.04 | 1.15 (0.40-3.33) | 0.03 | 0.02 | 1.42 (0.41-4.95) |
| | CT or TT vs. CC † | | | 0.75 (0.47-1.20) | | | 1.18 (0.80-1.80) |
| | TT vs. CC or CT ‡ | | | 1.26 (0.44-3.62) | | | 1.35 (0.39-4.69) |

*rs number refers to the National Center for Bioinformatics dbSNP build 127 reference sequence number.
†Dominant genetic model.
‡Recessive genetic model.



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**Table 4. IORs between *IL2* haplotypes and cigarette smoking on cervical and vulvar cancer risk based on a log-additive model**

| Haplotype* | Cervical cancer cases | | | Vulvar cancer cases | | |
|---|---|---|---|---|---|---|
| | Haplotype frequency | | IOR (95% CI) | Haplotype frequency | | IOR (95% CI) |
| | Smokers | Nonsmokers | | Smokers | Nonsmokers | |
| TTCC | 0.37 | 0.33 | 1.00 | 0.32 | 0.32 | 1.00 |
| G**C**GCC | 0.28 | 0.28 | 0.93 (0.63-1.36) | 0.32 | 0.25 | 1.34 (0.94-1.92) |
| **T**GCT | 0.15 | 0.18 | 0.81 (0.52-1.26) | 0.18 | 0.16 | 1.16 (0.79-1.71) |
| TGC**C** | 0.13 | 0.14 | 0.89 (0.57-1.41) | 0.11 | 0.16 | 0.71 (0.47-1.07) |
| TG**T**C | 0.07 | 0.07 | 0.90 (0.49-1.65) | 0.07 | 0.10 | 0.65 (0.39-1.07) |

*Alleles in each haplotype are listed from 5′ to 3′ (rs2069762, rs2069763, rs2069777, and rs2069778). Variant alleles are underlined.

cases (OR, 1.42; 95% CI, 1.00-2.03). Compared with the rs2069763 GG genotype, the TT genotype was associated with a statistically significant decreased risk of vulvar cancer (OR, 0.45; 95% CI, 0.27-0.76) that was essentially the same when the analysis was restricted to HPV-positive cases, and a marginally significant decreased risk of cervical cancer (OR, 0.60; 95% CI, 0.35-1.04). The ORs for cervical or vulvar cancer did not deviate significantly from the null for any of the other tagSNPs, nor were there substantial differences in ORs when the analyses were restricted to HPV-positive vulvar cancer cases.

## Discussion

Cigarette smoking is clearly an important risk factor for cervical and vulvar SCCs, but the mechanism underlying the association is unknown. To our knowledge, this is the first investigation into the effect modification of cigarette smoking by genetic variation in a T lymphocyte–regulatory cytokine as a pathway to explain part of the increased risk.

Prior studies have observed the presence of nicotine, cotinine, and other constituents of cigarette smoke and their metabolites in the cervical mucus of smokers (42, 43). These components have been shown to depress populations of cervical Langerhans cells and T lymphocytes (43, 44); cells that both produce and bind IL2. IL2 plays a critical role in propagating a Th1-mediated immune response, which is key in combating genital HPV infection and associated neoplasms (13-16). Furthermore, smokers have a near 2-fold decrease in IL2 concentration in cervical secretions, compared with nonsmokers (45). Studies of non–cervical-derived T lymphocytes have found that components of cigarette smoke, such as nicotine and hydroquinone, inhibit IL2 production (20-24). Genetic variation in *IL2* may have subtle effects on *IL2* transcription or protein structure that could influence concentrations or receptor binding (25), and potentially, these phenotypes could be exacerbated when IL2 production is impaired by smoking. The joint effect of genetic variation and cigarette smoking could conceivably influence the ability of IL2 to function normally, thereby increasing cancer risk.

In our study, the joint effect of the G allele of tagSNP rs2069762 and cigarette smoking on vulvar cancer risk was nearly 2-fold greater than expected under the multiplicative model. Although there was a suggestion of an increased vulvar cancer risk associated with

**Table 5. IORs between IL-2 diplotypes and cigarette smoking on cervical and vulvar cancer risk**

| Diplotype* | Cervical cancer cases | | | Vulvar cancer cases | | |
|---|---|---|---|---|---|---|
| | Diplotype frequency | | IOR (95% CI) | Diplotype frequency | | IOR (95% CI) |
| | Smokers | Nonsmokers | | Smokers | Nonsmokers | |
| TTCC/GGCC | 0.18 | 0.22 | 1.00 | 0.25 | 0.20 | 1.00 |
| T**T**CC/**T**TCC | 0.17 | 0.10 | 2.08 (1.01-4.30) | 0.08 | 0.08 | 0.85 (0.41-1.78) |
| TTCC/T**G**CT | 0.08 | 0.12 | 0.83 (0.37-1.87) | 0.11 | 0.11 | 0.82 (0.43-1.56) |
| TTCC/TGC**C** | 0.11 | 0.08 | 1.69 (0.76-3.79) | 0.07 | 0.08 | 0.74 (0.36-1.51) |
| T**G**CT/GGCC | 0.08 | 0.09 | 1.09 (0.47-2.53) | 0.13 | 0.05 | 2.09 (0.98-4.46) |
| GGC**C**/G**G**CC | 0.09 | 0.07 | 1.68 (0.72-3.95) | 0.07 | 0.06 | 0.97 (0.44-2.13) |
| **T**GCC/G**G**CC | 0.05 | 0.06 | 1.05 (0.41-2.72) | 0.05 | 0.08 | 0.55 (0.26-1.17) |
| TTCC/T**G**TC | 0.03 | 0.05 | 0.72 (0.22-2.34) | 0.04 | 0.09 | 0.37 (0.16-0.85) |
| T**C**TC/GG**C**C | 0.06 | 0.04 | 1.70 (0.63-4.58) | 0.05 | 0.03 | 1.25 (0.47-3.33) |
| TGCT/T**G**CC | 0.03 | 0.06 | 0.65 (0.20-2.08) | 0.03 | 0.08 | 0.37 (0.15-0.87) |
| TGC**T**/TGCT | 0.04 | 0.03 | 1.66 (0.54-5.11) | 0.02 | 0.02 | 0.95 (0.26-3.47) |
| TGC**T**/TGT**C** | 0.03 | 0.02 | 2.28 (0.55-9.37) | 0.03 | 0.04 | 0.53 (0.19-1.45) |
| TGC**C**/TGCC | 0.02 | 0.02 | 1.65 (0.38-7.19) | 0.02 | 0.02 | 0.72 (0.20-2.55) |
| TGCC/TGTC | 0.02 | 0.04 | 0.77 (0.20-3.04) | 0.01 | 0.03 | 0.35 (0.10-1.22) |

*The two haplotypes carried on each chromosome are separated by the ''/''. Alleles in each haplotype are listed from 5′ to 3′ (rs2069762, rs2069763, rs2069777, and rs2069778). Variant alleles are underlined.



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**Table 6. Main effect of each tagSNP on cervical and vulvar cancer risk**

| TagSNP* | Genotype | OR (95% CI) † | |
|---|---|---|---|
| | | Cervical cancer | Vulvar cancer |
| rs2069762 | TT | 1.00 | 1.00 |
| | TG | 1.14 (0.79-1.66) | 1.28 (0.92-1.78) |
| | GG | 1.15 (0.57-2.29) | 0.84 (0.47-1.50) |
| rs2069763 | GG | 1.00 | 1.00 |
| | GT | 0.78 (0.53-1.15) | 0.97 (0.69-1.37) |
| | TT | 0.60 (0.35-1.04) | 0.45 (0.27-0.76) |
| rs2069777 | CC | 1.00 | 1.00 |
| | CT | 1.12 (0.66-1.89) | 1.33 (0.84-2.11) |
| rs2069778 | CC | 1.00 | 1.00 |
| | CT | 0.87 (0.58-1.30) | 1.03 (0.73-1.47) |
| | TT | 1.31 (0.47-3.69) | 0.67 (0.27-1.71) |

*rs number refers to the National Center for Bioinformatics dbSNP build 127 reference sequence number.
†All controls were seropositive for HPV16 or HPV18 L1 antibodies.

heterozygosity for rs2069762, the possibility that this was a spurious finding is supported by the observation of a reduced risk of similar magnitude associated with homozygosity for the G allele. The haplotype containing the variant allele of rs2069762, GGCC, was also associated with a supramultiplicative joint effect with smoking. The IOR for the diplotype carrying two copies of the variant allele of rs2069762 (GGCC/GGCC), compared with the reference diplotype which had one copy of the variant allele (TTCC/GGCC), was nearly null. This result is consistent with the single locus model which suggested a dominant genetic effect (i.e., similar IORs for heterozygotes and homozygotes for the variant allele). The rs2069762 polymorphism is located in a 5′ flanking, evolutionarily conserved region of *IL2* (46, 47), and the variant allele has been associated with increased *IL2* transcription in cultured peripheral blood lymphocytes (25). Based on these limited experimental data, one might expect carriers of the variant allele (putative high IL2 producers) to have a stronger T lymphocyte–mediated immune response, and thus decreased risk of HPV-related cancer, and in combination with smoking, either no multiplicative effect on risk of HPV-related cancers, or potentially even a submultiplicative effect. Alternatively, the putative high IL2–producing variant allele of rs2069762 may contribute to a positive interaction with cigarette smoking in vulvar cancer risk through an inflammatory pathway. A positive association between inflammation and vulvar cancer risk has been shown previously (48), and the high-producer *IL2* genotype could conceivably lead to an unregulated and unfavorable inflammatory response to HPV infection in vulvar tissue when coupled with cigarette smoking (49). The putative dampening effect of cigarette smoke on IL2 levels may be outweighed by the tumor-promoting potential of cigarette smoking which has been linked to the induction of the proinflammatory transcription factor nuclear factor κB (50) and inhibition of apoptosis (51). Thus, although no consistent main effect of the rs2069762 was observed, it is conceivable that the joint effect of rs2069762 and cigarette smoking would be important in vulvar cancer risk.

Our observation that the joint effect of rs2069762 and cigarette smoking was associated with a positive departure from multiplicativity in vulvar, but not cervical, cancer risk has no obvious explanation. However, as previously mentioned, the functional effects of cigarette smoking and genetic variation on IL2 concentrations have mostly been identified in healthy cervical tissue or peripheral blood, and thus, may not reflect the immune environment in vulvar tissue. Unfortunately, there are limited comparable data on cervical and vulvar HPV or cancer immunity. A few studies suggest that women with cervical and vulvar high-grade lesions elicit a similar T lymphocyte responses to HPV (52, 53). In contrast, a study of HPV16-positive high-grade vulvar lesions and cervical cancer reported site-specific associations with polymorphisms of class I and class II human leukocyte antigens (54), loci that play an important role in regulating T-lymphocyte responses to viral proteins. Among the cases and HPV-seropositive controls included in this current study, the age-, sex partner-, parity-, and education-adjusted OR for current smoking in cervical cancer risk was 1.48 (95% CI, 0.99-2.22); in vulvar cancer, the OR was 3.97 (95% CI, 2.73-5.79). These data, together with prior observations that cervical and vulvar cancer differ in strength of association with cigarette smoking (2-5), suggest that the mechanism of smoking-related carcinogenesis may differ between sites. Furthermore, the proportion of current smokers who were heavy smokers (≥1 pack per day) was similar for cervical cancer (64%) and vulvar cancer (60%) cases, and restricting the analyses for rs2069762 to heavy smokers did not substantially influence the IORs. These data add further support to the notion that there may be biological, possibly immunologic, differences between the two sites that influence smoking-related carcinogenesis, not simply differences in smoking habits. Lastly, the observed statistically significant joint effect of rs2069762 and cigarette smoking in vulvar cancer risk may be a false-positive finding.

Rs2069762 was not in linkage disequilibrium with any other *IL2* SNPs among Caucasians in the SeattleSNPs project, which reduces but does not eliminate the possibility that the interaction we observed was due to linkage with other loci. In the greater 40-kbp region encompassing *IL2*, the International HapMap Project (55) shows linkage between the *IL2* rs2069762 polymorphism and three 3′ flanking polymorphisms in the testis nRNA-binding protein gene (*TENR*, rs716501, rs17454584, and rs4833826), ~20 kbp 3′ of *IL2*. Little is known regarding tissue-specific expression of *TENR* in humans; however, in mice, *TENR* is exclusively expressed in the testis, thus an influence of these polymorphisms on vulvar cancer risk is highly unlikely (56).

Carriership of two copies of the variant allele of rs2069763, a synonymous SNP, was associated with an at least 1.66-fold excess joint effect with cigarette smoking in cervical cancer risk in the single locus and diplotype models. Additionally, homozygosity for the variant allele of rs2069763 was associated with a reduced risk of cervical and vulvar cancer. Although nothing is currently known regarding the phenotypic consequences of this tagSNP, located in a highly conserved region of *IL2*, there is growing evidence that ''silent'' polymorphisms may

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elicit effects through subtle alterations in transcription or mRNA transport (57, 58). Furthermore, in the SeattleSNP project, this tagSNP was in linkage disequilibrium with an intronic SNP (rs2069772) proximal (~100 bp) to the intron three-exon four junction and could potentially alter splice factor binding. The observed reduced risk of cervical cancer associated with rs2069763 is seemingly at odds with the observation of a greater than multiplicative joint effect of rs2069763 and cigarette smoking in cervical cancer risk. These observations may be reconciled by the delicate immune balance between immunoregulation and inflammation in response to HPV infection and associated neoplastic changes. It is conceivable that the variant allele of rs2069772 is associated with reduced cervical and vulvar cancer risk via increased IL2 activity, and thus, an effective regulatory T-lymphocyte response against HPV and emerging cancer cells. However, in the context of a tumor-promoting environment associated with cigarette smoking as described above, a highly effective regulatory T-lymphocyte response may be shifted towards an unregulated inflammatory response, providing a mechanism for carcinogenesis (59). The lack of a joint effect between rs2069763 and cigarette smoking in vulvar cancer risk may reflect differences between the immune responses in these tissues.

It is apparent by the *IL2* haplotypes inferred from our genotyping data that our study population exhibited a similar pattern of linkage disequilibrium to that of the SeattleSNP population, from which our tagSNP selection was based. As each of our haplotypes was uniquely marked by a tagSNP variant allele, our haplotype models are essentially the same as log-additive single locus models. In contrast, the results from our diplotype analysis have the potential to identify joint influences of haplotypes. Carriership of one of three diplotypes together with cigarette smoking was associated with either a supramultiplicative (TGCT/GGCC) or submultiplicative (TTCC/TGTC or TGCT/TGCC) joint effect in vulvar cancer risk. Due to the rarity of these diplotypes, it is possible that the observed interaction is an artifact of small numbers. Alternatively, the interaction of alleles on separate haplotypes may influence IL2 production or function in some unknown way. The paucity of data regarding the functional consequences of these alleles makes it difficult to speculate on the biological effect of a potential interaction of alleles.

Our decision to use HPV-seropositive controls for our analysis of independence and assessment of main effects ultimately influences the interpretation of the results. Immune system factors may influence HPV-associated cancer risk during (at least) three stages of disease progression: (*a*) upon initial HPV exposure, (*b*) during the establishment of a persistent HPV infection, and (*c*) during neoplastic progression. Seropositive controls were women who had mounted an immune response to HPV, however, a proportion of these women may have developed a persistent infection whereas others may have encountered and cleared an infection. Furthermore, there is the possibility that women may have been exposed to an HPV infection, but did not mount an immune response and thus are not included in our control group. Our choice to include seropositive controls allows us to examine the role of IL-2 variants in the stages of disease progression beyond the initial mounting of an immune response to an HPV infection. Because the motivation for this study was to investigate a potential mechanism for current cigarette smoking, these controls allow us to focus on the later stages of disease progression in which current cigarette smoking is most likely relevant. Unfortunately, we do not have cancer-free individuals with persistent genital HPV-infection defined by HPV DNA status in our study, therefore, we cannot separate our inferences regarding the joint effect of *IL2* variants and cigarette smoking, or *IL2* variants alone, on HPV persistence and tumor progression.

We chose a case-only design because it offers several advantages, including high statistical power, for exploring the role of *IL2* variation as a pathway to explain the increased risk of cervical and vulvar cancer associated with cigarette smoking. Although case-only studies are generally more powerful than case-control studies for detecting departures from multiplicative joint effects, they are still susceptible to sources of systematic error, which could lead to spurious results (60, 61). For example, selection bias could occur if a case's inclusion in this study was related jointly to her smoking status and *IL2* genotype, although this seems unlikely given that decisions to participate or provide a blood sample are made in the absence of knowledge of one's genetic makeup. Similarly, recall of information on smoking by cases is not likely to be dependent on genotype. Therefore, misclassification of smoking status will most likely be nondifferential, and if present, would bias the IOR towards the null. Another limitation of the case-only study is that it can only assess effect modification on a multiplicative, as opposed to additive, scale.

The strengths of this study include the population-based recruitment of cases (and controls), attempted coverage of all common genetic variation in *IL2*, and the use of single- and multi-locus analytic methods. Furthermore, the assumption of conditional independence between the genotypes of each tagSNP and cigarette smoking in the HPV-exposed population from which the cervical and vulvar cases arise is an important foundation for this study, and we found this assumption to hold in a large sample of HPV-seropositive controls.

IL2 is central to T lymphocyte immune response, but by no means is it the only influential cytokine or immune factor to potentially modulate the effect of cigarette smoking in cervical or vulvar cancer risk. For example, cervical cancer risk is reduced among carriers of the human leukocyte antigen class II DRB1*13/DBQ1*0603 alleles (62), and possibly, certain polymorphisms in genes coding for IFN-γ (63) and interleukin 10 (64). The possibility that these polymorphisms, or polymorphisms of other cytokines, receptors, or immune factors, modify the association between cigarette smoking and cancer risk has yet to be explored.

Substantial progress in recent years towards the development and uptake of prophylactic HPV vaccines provides hope for reducing the burden of HPV infection and associated neoplasms in the future (65). Nonetheless, there remain a large number of women that will not benefit from the vaccine as they have already acquired HPV infection, are beyond the target age of vaccination, or live in low-resource regions of the world that are challenged by the high cost and distribution of a vaccine

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(66, 67). Identification of gene-environment interactions that contribute to cervical and vulvar cancer risk may help shed light on the biological mechanisms leading to cancer, and potentially identify women who are at increased risks of developing these malignancies.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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