

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

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23 **Abstract**

24 Cigarette smoking is an established co-factor to human papillomavirus (HPV) in the development of
25 cervical and vulvar squamous cell carcinoma (SCC), and may influence risk through an
26 immunosuppressive pathway. Genetic variation in interleukin 2 (*IL2*), associated in some studies with
27 inhibition of HPV-targeted immunity, may modify the effect of smoking on the risk of HPV-related
28 anogenital cancers. We conducted a population-based case-only study to measure the departure from a
29 multiplicative joint effect of cigarette smoking and *IL2* variation on cervical and vulvar SCC.
30 Genotyping of four *IL2* tagSNPs (rs2069762, rs2069763, rs2069777, and rs2069778) was performed in
31 399 cervical and 486 vulvar SCC cases who had been interviewed regarding their smoking history.
32 Compared to cases carrying the rs2069762 TT genotype, we observed significant departures from
33 multiplicativity for smoking and carriership of the TG or GG genotypes in vulvar SCC risk (interaction
34 odds ratio (IOR)=1.67, 95% confidence interval (CI): 1.16, 2.41). Carriership of one of three
35 diplotypes together with cigarette smoking was associated with either a supra-multiplicative
36 (TGCT/GGCC, IOR=2.09, 95% CI: 0.98, 4.46) or sub-multiplicative (TTCC/TGTC, IOR=0.37, 95%
37 CI: 0.16, 0.85 or TGCT/TGCC, IOR=0.37, 95% CI: 0.15, 0.87) joint effect in vulvar cancer risk. For
38 cervical SCC, departure from multiplicativity was observed for smokers homozygous for the
39 rs2069763 variant allele (TT versus GG or GT genotypes) (IOR=1.87, 95% CI: 1.00, 3.48), and for
40 carriership of the TTCC/TTCC diplotype, (IOR=2.08, 95% CI: 1.01, 4.30). These results suggest that
41 cervical and vulvar SCC risk among cigarette smokers is modified by genetic variation in *IL2*.

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43

44

45 **Introduction**

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

51 Cigarette smoking is among the most well-established HPV-co-factors in the etiology of these
52 malignancies (4). Current smokers are at approximately two- to three-fold increased cervical squamous
53 cell carcinoma (SCC) risk (5), and greater than three-fold vulvar SCC risk (2, 3), whereas former
54 cigarette smokers tend to be at little or no increased risk (5, 6). Studies have also observed an
55 association of cervical SCC risk with increasing duration of smoking (5, 7), although this trend appears
56 to be driven by the high proportion of long-term smokers who are also current smokers (7).
57 Experimental evidence linking smoking cessation and a decrease in cervical lesion size (8) also
58 highlights the important role of current cigarette smoking in cervical SCC risk.

59 The biological mechanism whereby cigarette smoking increases cervical and vulvar SCC risk
60 remains largely undetermined (9). One possibility is that smoking enhances immunosuppression (8).
61 The importance of the adaptive immune response in HPV-associated cancer risk is emphasized by
62 studies showing that HIV-infected women have a substantially increased risk of developing cervical
63 and vulvar cancer (10, 11), and women with drug-induced immunosuppression are nine times more
64 likely than the general population to develop an HPV infection, and 16 times more likely to develop
65 cervical cancer (12). In immunocompetent patients capable of preventing persistent HPV infection and
66 related neoplastic changes, Th1 cytokines such as interleukin 2 (IL-2) propagate a T lymphocyte-
67 mediated immune response to HPV and tumor antigens (13-16). IL-2 is a T lymphocyte derived

68 cytokine that is secreted minutes after activation of a T lymphocyte receptor by an antigen bound to a
69 major histocompatibility complex receptor on an antigen presenting cell. IL-2 acts in an autocrine
70 manner by binding the IL-2 receptor on activated T lymphocytes and inducing transcription of other
71 Th1 cytokines, which together propagate the T lymphocyte response (17). IL-2 is considered to be a
72 key component of the adaptive immune response to HPV infection and the development and growth of
73 tumors driven by the viral oncogenes (18, 19).

74 Experimental studies demonstrate an influence of both cigarette smoking (20-24) and genetic
75 variation (25) on IL-2 expression, suggesting the possibility that cigarette smoking and inherited
76 genetic variation in *IL2* interact to increase cervical and vulvar SCC risk. We conducted the present
77 study to test that hypothesis.

78

79 **Methods**

80 **Study design**

81 Assessing the joint effect of cigarette smoking and *IL2* nucleotide variation on HPV-dependent
82 cancers would ideally involve assessing the interaction effect among women who have persistent
83 oncogenic HPV infection (26). Practically, however, oncogenic HPV infection in the general
84 population of adult women identified with current detection methods is uncommon (between 2 and
85 12%), and persistent infection is rare (27). A case-only design avoids the difficult task of selecting a
86 control group with persistent HPV infection. Under the assumption of independence between cigarette
87 smoking and variation in *IL2*, the interaction odds ratio (IOR) from a case-only design provides an
88 estimate of effect modification equivalent to that derived from a case-control study under a
89 multiplicative model (28). In addition, the case-only design offers higher precision to estimate the IOR
90 compared to a standard case-control design (29).

91 **Study population**

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

111

112 **Data and specimen collection**

113 In the case-control study, in-person interviews were conducted to elicit information on
114 demographic and other characteristics with a known or suspected relationship to anogenital cancer,
115 including cigarette smoking. A woman was considered a smoker if she reported smoking 100 or more
116 cigarettes in her lifetime. Venous blood samples were drawn at the time of the interview to provide
117 serum samples for HPV 16 and 18 antibody testing as described previously (34). Beginning in 1991,
118 five years after the start of the study, we expanded the blood collection to include samples from which
119 DNA could be isolated. We also recontacted cervical, but not vulvar, cancer cases interviewed in the
120 earliest years of the study and asked them to provide these additional blood samples. A small
121 proportion of study participants (3%) preferred to donate a buccal cell sample, which was collected
122 using a standardized oral rinse procedure, in place of blood. We attempted to retrieve archival tissue
123 blocks from biopsy or surgery to determine the presence and type of HPV DNA in the tumors of the
124 cervical and vulvar cancer cases. HPV DNA typing on tumor tissue was performed using polymerase
125 chain reaction (PCR) methods, as described in detail previously (35).

126

127 **Response Rates**

128 Among the 1,189 eligible cervical SCC patients identified for the parent case-control study,
129 744 (62.6%) were interviewed and among those interviewed 674 (90.6%) provided a specimen from
130 which DNA could be obtained. A similar proportion, 67.6%, (807 of the 1194 eligible vulvar SCC
131 cases) were interviewed, however, specimens from which DNA could be obtained were only collected
132 from 73.4% of participating vulvar cancer cases. This percentage is largely affected by the fact that, as
133 described above, the early version of the parent study protocol did not include collection of blood
134 specimens from which DNA could be isolated, and that and the vulvar cancer cases, unlike the cervical
135 cancer cases, were not reapproached for these specimens once the protocol was changed. Reasons for

136 non-participation were largely similar for the two cancers and included doctor refusal to allow us to
137 contact the patient (5% and 6%, for cervical and vulvar cases respectively), refusal of the patient to
138 participate or our inability to locate the patient (22% and 24%), or patient death (10% and 3%). Drawn
139 from the Caucasian participants who had a sufficient DNA sample at the time of this study, our
140 analyses included 399 cervical and 490 (434 *in situ*) vulvar SCC cases. Four vulvar SCC cases (3 *in*
141 *situ*) were not included in any of the tables because a genotyping result could not be obtained from
142 their samples for any of the polymorphisms included in this study, resulting in a total of 486 vulvar
143 SCC cases. Sixty-three % (n=251) of cervical cancer cases and 71% (n=347) of vulvar cancer cases
144 included in this study had tumor tissue available that had been tested for HPV DNA. Sixty-seven % of
145 eligible control women agreed to participate, and 83.9% (N=1,372) of those interviewed donated a
146 blood sample from which DNA could be obtained.

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

153

154 **TagSNP selection**

155 Information on *IL2* nucleotide variation was obtained from the SeattleSNPs Variation
156 Discovery Resource (36), <http://pga.gs.washington.edu/data/il2/>. Briefly, SeattleSNPs has resequenced
157 exons, introns, and 1000 bp or more on the 5' and 3' ends of each target gene in DNA from 23 Centre
158 d'Etude du Polymorphisme Humain (CEPH) parents of European descent and 24 African-Americans,

159 obtained from the Coriell Repository (Camden, NJ). Using the European descent data, all SNPs with a
160 variant allele frequency of at least 5% were identified; seven out of the ten SNPs met this criterion.
161 Next, a pairwise r^2 cutpoint of 0.80 was used to delineate groups of highly correlated SNPs (37) and
162 one polymorphism (i.e. tagSNPs) per group was selected to be genotyped. When more than one
163 possible tagSNP for a particular group of correlated SNPs was identified, information regarding
164 putative function reported in the literature and location of the SNP informed tagSNP selection. The
165 National Center for Bioinformatics (NCBI) dbSNP build 127 reference sequence number for the four
166 selected *IL2* tagSNPs are rs2069762, rs2069763, rs2069777, and rs2069778.

167

168 **Genotyping of *IL2* tagSNPs**

169 Genomic DNA was extracted from buffy coat aliquots from blood samples, or cell pellets from
170 buccal samples, using a phenol chloroform method (38). Genotyping was performed using Pre-
171 Designed or Custom TaqMan® genotyping assays from Applied Biosystems following manufacturer's
172 protocol (Applied Biosystems, Foster City, CA). Briefly, the assays were conducted in a 5 μ l volume
173 containing 5 to 50 ng genomic DNA, 2.5 μ l of the 2x Universal Master Mix with uracil-DNA
174 glucosylase 200 nM of each assay-specific primer and 900 nM of each assay-specific FAM and VIC
175 fluorescently labeled probe. Reactions were amplified using a 9700 PCR machine or a 7500 Real-Time
176 PCR system (Applied Biosystems, Foster City, CA) for 50°C for 2 min, 95°C 10 min followed by 40 to
177 50 cycles of 92°C for 15 to 30 s and 58 to 60°C for 1 to 1.5 min. The fluorescence release was
178 measured by the 7500 Real-Time PCR system using the allelic discrimination setting of the Sequence
179 Detection Software version 1.2.3 (Applied Biosystems). Probe and primer sequences are listed in
180 Supplementary Table 1. Two to three positive controls (samples known to be heterozygous or

181 homozygous for each allele based on sequencing) and negative controls (wells containing no DNA)
182 were included in each reaction plate. Specimens were organized so that the replicate QC DNA
183 aliquots, which comprised approximately 10% of the specimens, were distributed throughout the
184 reaction plates. Analysis of these replicates revealed a low discordance proportion of 1%. Laboratory
185 personnel were blinded to all research information about the samples, including the identities of the
186 QC replicate aliquots.

187

188 **Data analysis**

189 TagSNP genotypes were tested for consistency with Hardy-Weinberg equilibrium (HWE)
190 within the HPV seropositive control sample using a Pearson's χ^2 *p* value cutpoint of 0.05. The control
191 sample was also used to test for independence of smoking status and *IL2* tagSNP genotypes. One
192 approach to test for independence is to use logistic regression to model smoking as a dependent
193 variable and genotype as an independent variable among the controls. Alternatively, Umbach and
194 Weinberg (1997) proposed a method which offers higher precision that uses a likelihood ratio test
195 (LRT) to compare two nested log-linear models for each tagSNP (39). In the full model, the logarithm
196 of the expected cell count was the dependent variable that fully parameterizes the joint effect of
197 cigarette smoking and tagSNP genotypes separately for cases and controls. The reduced model fixed
198 the joint effect parameter for the controls at zero. Thus the LRT comparing these two models is a test
199 of the association between tagSNP genotypes and cigarette smoking among controls. An LRT *p* value
200 of 0.05 or less, or an exponentiated joint effect parameter for cigarette smoking and tagSNP genotype
201 among controls (The OR from the full model) departing substantially from the null, was taken as
202 evidence of a statistically significant lack of independence between cigarette smoking status and *IL2*

203 genotypes. For the cervical cancer analyses, these models were fit after excluding 56 controls without
204 intact uteri, resulting in 180 controls.

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

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

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

232

233 **Results**

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

241 TagSNP variant allele frequencies ranged from 0.07 to 0.38 (Table 2). We did not find
242 statistical evidence of lack of fit to HWE for any of the tagSNPs. We observed independence of
243 tagSNP genotypes and cigarette smoking among both cervical and vulvar HPV seropositive control
244 groups, as indicated by ORs close to the null value and LRT *p* values ≥ 0.05 (Table 2).

245 Compared to homozygous carriers of the common allele of tagSNP rs2069762 (TT genotype),
246 positive departures from multiplicativity were observed for vulvar cancer cases carrying one
247 (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.

248 The dominant genetic model showed a similar departure for smokers carrying either the TG or GG
249 genotypes, versus carriers of the TT genotype (IOR=1.67, 95% CI: 1.16, 2.41). A similar departure
250 from multiplicativity was observed when the analysis was restricted to the oncogenic HPV DNA
251 positive or HPV16 or HPV18 L1 seropositive vulvar cancer cases, TG or GG genotypes versus TT,
252 IOR=1.92, 95% CI: 1.21, 3.04. However, a slightly increased IOR was observed for all women who
253 were tested for either tumor HPV DNA or HPV serology (N=363, IOR=1.83, 95% CI: 1.20, 2.79)
254 compared to women who did not have tumor tissue available for testing (N=123, IOR=1.25, 95% CI:
255 0.59, 2.67).

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

261 We observed five haplotypes in *IL2*, each uniquely tagged by the presence of a single variant
262 allele, TTCC, GGCC, TGCT, and TGTC, or no variant alleles, TGCC (as indicated by the underlined
263 allele), Table 4. Compared to carriers of the most common haplotype, TTCC, cigarette smoking and
264 carriership of any other haplotype did not result in significant departures from multiplicativity in either
265 cervical or vulvar cancer risk. The GGCC haplotype, defined by the variant allele of rs2069762, was
266 associated with a positive, but not statistically significant, departure from multiplicativity in vulvar
267 cancer risk (IOR=1.34, 95% CI: 0.94, 1.92). Compared to carriers of the most common diplotype
268 (TTCC/GGCC), carriership of the TTCC/TTCC diplotype, defined by two copies of the variant allele
269 of rs2069763, and cigarette smoking together resulted in a positive departure from multiplicative joint
270 effects on cervical (IOR=2.08, 95% CI: 1.01, 4.30), but not vulvar (IOR=0.85, 95% CI: 0.41, 1.78),

271 cancer risk (Table 5). The second most common diplotype among smokers with cervical cancer,
272 TTCC/TTCC, defined by two copies of the variant allele of rs2069763, together with cigarette
273 smoking was associated with a significant positive two-fold departure from multiplicatively in cervical
274 cancer risk (IOR=2.08, 95% CI: 1.01, 4.30), compared to the reference diplotype, TTCC/GGCC.
275 Similarly, TGCT/GGCC, a common diplotype among vulvar cancer cases, was associated with a
276 marginally significant positive two-fold departure from multiplicatively in vulvar cancer risk
277 (IOR=2.09, 95% CI: 0.98, 4.46). Two rare diplotypes were associated with sub-multiplicative joint
278 effects in vulvar cancer risk, TTCC/TGTC, IOR=0.37, 95% CI: 0.16, 0.85, and TGCT/TGCC,
279 IOR=0.37, 95% CI: 0.15, 0.87.

280 The ORs for the main effect of each tagSNP on cervical and vulvar cancer risk are presented in
281 Table 6. Compared to the rs2069762 TT genotype, the TG genotype was associated with a marginally
282 significant increased risk of vulvar cancer (OR=1.28, 95% CI: 0.92-1.78), which was slightly more
283 pronounced when the analysis was restricted to HPV positive vulvar cancer cases (OR=1.42, 95% CI:
284 1.00-2.03). Compared to the rs2069763 GG genotype, the TT genotype was associated with a
285 statistically significant decreased risk of vulvar cancer (OR=0.45, 95% CI: 0.27-0.76) that was
286 essentially the same when the analysis was restricted to HPV positive cases, and a marginally
287 significant decreased risk of cervical cancer (OR=0.60, 95% CI: 0.35-1.04). The ORs for cervical or
288 vulvar cancer did not deviate significantly from the null for any of the other tagSNPs, nor were there
289 substantial differences in ORs when the analyses were restricted to HPV positive vulvar cancer cases.

290

291 **Discussion**

292 Cigarette smoking is clearly an important risk factor for cervical and vulvar SCCs, but the
293 mechanism underlying the association is unknown. To our knowledge, this is the first investigation

294 into effect modification of cigarette smoking by genetic variation in a T lymphocyte regulatory
295 cytokine as a pathway to explain part of the increased risk.

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

309 In our study, the joint effect of the G allele of tagSNP rs2069762 and cigarette smoking on
310 vulvar cancer risk was nearly two-fold greater than expected under the multiplicative model. While
311 there was a suggestion of an increased vulvar cancer risk associated with heterozygosity for
312 rs2069762, the possibility that this was a spurious finding is supported by observation of a reduced risk
313 of similar magnitude associated with homozygosity for the G allele. The haplotype containing the
314 variant allele of rs2069762, GGCC, was also associated with a supra-multiplicative joint effect with
315 smoking. The IOR for the diplotype carrying two copies of the variant allele of rs2069762,
316 GGCC/GGCC, compared to the reference diplotype which had one copy of the variant allele,

317 TTCC/GGCC, was nearly null. This result is consistent with the single locus model which suggested a
318 dominant genetic effect (i.e. similar IORs for heterozygotes and homozygotes for the variant allele).
319 The rs2069762 polymorphism is located in a 5' flanking, evolutionarily conserved, region of *IL2* (46,
320 47), and the variant allele has been associated with increased IL-2 transcription in cultured peripheral
321 blood lymphocytes (25). Based on these limited experimental data, one might expect carriers of the
322 variant allele (putative high IL-2 producers) to have a stronger T lymphocyte mediated immune
323 response, and thus decreased risk of HPV-related cancer, and in combination with smoking, either no
324 multiplicative effect on risk of HPV-related cancers, or potentially even a sub-multiplicative effect.
325 Alternatively, the putative high IL-2-producing variant allele of rs2069762 may contribute to a positive
326 interaction with cigarette smoking in vulvar cancer risk through an inflammatory pathway. A positive
327 association between inflammation and vulvar cancer risk has been shown previously (48), and the
328 high-producer IL-2 genotype could conceivably lead to an unregulated and unfavorable inflammatory
329 response to HPV infection in vulvar tissue when coupled with cigarette smoking (49). The putative
330 dampening effect of cigarette smoke on IL-2 levels may be outweighed by the tumor promoting
331 potential of cigarette smoking which has been linked to the induction of the pro-inflammatory
332 transcription factor NF-KB (50) and inhibition of apoptosis (51). Thus, while no consistent main effect
333 of the rs2069762 was observed, it is conceivable the joint effect of rs2069762 and cigarette smoking
334 would be important in vulvar cancer risk.

335 Our observation that the joint effect of rs2069762 and cigarette smoking was associated with a
336 positive departure from multiplicativity in vulvar, but not cervical, cancer risk has no obvious
337 explanation. However, as previously mentioned, functional effects of cigarette smoking and genetic
338 variation on IL-2 concentrations have mostly been identified in healthy cervical tissue or peripheral
339 blood, and thus may not reflect the immune environment in vulvar tissue. Unfortunately, there are

340 limited comparable data on cervical and vulvar HPV or cancer immunity. A few studies suggest that
341 women with cervical and vulvar high grade lesions elicit a similar T lymphocyte responses to HPV
342 (52, 53). In contrast, a study of HPV-16 positive high grade vulvar lesions and cervical cancer reported
343 site-specific associations with polymorphisms of class I and II human leukocyte antigens (HLA) (54),
344 loci that play an important role in regulating T lymphocyte responses to viral proteins. Among the
345 cases and HPV seropositive controls included in this current study, the age-, sex partner-, parity-, and
346 education-adjusted OR for current smoking in cervical cancer risk was 1.48 (95% CI: 0.99-2.22); in
347 vulvar cancer the OR was 3.97 (95% CI: 2.73-5.79). These data, together with prior observations that
348 cervical and vulvar cancer differ in strength of association with cigarette smoking (2-5), suggest that
349 the mechanism of smoking related carcinogenesis may differ between sites. Furthermore, the
350 proportion of current smokers who were heavy smokers (≥ 1 pack per day) was similar for cervical
351 cancer (64%) and vulvar cancer (60%) cases, and restricting the analyses for rs2069762 to heavy
352 smokers did not substantially influence the IORs. These data add further support to the notion that
353 there may be biological, possibly immunological, differences between the two sites that influence
354 smoking-related carcinogenesis, not simply differences in smoking habits. Lastly, the observed
355 statistically significant joint effect of rs2069762 and cigarette smoking in vulvar cancer risk may be a
356 false positive finding.

357 Rs2069762 was not in linkage disequilibrium with any other *IL2* SNPs among Caucasians in
358 the SeattleSNPs project, which reduces but does not eliminate the possibility that the interaction we
359 observed was due to linkage with other loci. In the greater 40 kilo-bp region encompassing *IL2*, the
360 International HapMap Project (55) shows linkage between the *IL2* rs2069762 polymorphism and three
361 3' flanking polymorphisms in the testis nuclear RNA-binding protein gene (TENR, rs716501,
362 rs17454584, and rs4833826), approximately 20 kilo-bp 3' of *IL2*. Little is known regarding tissue-

363 specific expression of TENR in humans, however in mice TENR is exclusively expressed in the testis
364 thus an influence of these polymorphisms on vulvar cancer risk is highly unlikely (56).

365 Carriership of two copies of the variant allele of rs2069763, a synonymous SNP, was
366 associated with at least a 1.66-fold excess joint effect with cigarette smoking in cervical cancer risk in
367 the single locus and diplotype models. Additionally, homozygosity for the variant allele of rs2069763
368 was associated with reduced risk of cervical and vulvar cancer. Although nothing is currently known
369 regarding phenotypic consequences of this tagSNP, located in a highly conserved region of *IL2*, there
370 is growing evidence that “silent” polymorphisms may elicit effects through subtle alterations in
371 transcription or mRNA transport (57, 58). Furthermore, in the SeattleSNPs project, this tagSNP was in
372 linkage disequilibrium with an intronic SNP (rs2069772) proximal (~100 bp) to the intron three-exon
373 four junction and could feasibly alter splice factor binding. The observed reduced risk of cervical
374 cancer associated with rs2069763 is seemingly at odds with the observation of a greater than
375 multiplicative joint effect of rs2069763 and cigarette smoking in cervical cancer risk. These
376 observations may be reconciled by the delicate immune balance between immunoregulation and
377 inflammation in response to HPV infection and associated neoplastic changes. It is conceivable that the
378 variant allele of rs2069772 is associated with reduced cervical and vulvar cancer risk via increased IL-
379 2 activity and thus an effective regulatory T-lymphocyte response against HPV and emerging cancer
380 cells. However, in the context of a tumor-promoting environment associated with cigarette smoking as
381 described above, a highly effective regulatory T-lymphocyte response may be shifted towards an
382 unregulated inflammatory response, providing a mechanism for carcinogenesis (59). The lack of a joint
383 effect between rs2069763 and cigarette smoking in vulvar cancer risk may reflect differences between
384 the immune responses in these tissues.

385 It is apparent by the *IL2* haplotypes inferred from our genotyping data that our study population
386 exhibited a similar pattern of linkage disequilibrium to that of the SeattleSNPs population, from which
387 our tagSNP selection was based. As each of our haplotypes was uniquely marked by a tagSNP variant
388 allele, our haplotype models are essentially the same as log-additive single locus models. In contrast,
389 the results from our diplotype analysis have the potential to identify joint influences of haplotypes.
390 Carriership of one of three diplotypes together with cigarette smoking was associated with either a
391 supra-multiplicative (TGCT/GGCC) or sub-multiplicative (TTCC/TGTC or TGCT/TGCC) joint effect
392 in vulvar cancer risk. Due to the rarity of these diplotypes, it is possible that the observed interaction is
393 an artifact of small numbers. Alternatively, the interaction of alleles on separate haplotypes may
394 influence IL-2 production or function in some unknown way. The paucity of data regarding of
395 functional consequences of these alleles makes it difficult to speculate on the biological effect of a
396 potential interaction of alleles.

397 Our decision to use HPV seropositive controls for our analysis of independence and assessment
398 of main effects ultimately influences the interpretation of the results. Immune system factors may
399 influence HPV-associated cancer risk during (at least) three stages of disease progression: 1) upon
400 initial HPV exposure, 2) during the establishment of a persistent HPV infection, and 3) during
401 neoplastic progression. Seropositive controls are women who have mounted an immune response to
402 HPV, however a proportion of these women may have developed a persistent infection while others
403 may have encountered and cleared an infection. Furthermore, there is the possibility that women may
404 have been exposed to an HPV infection, but did not mount an immune response and thus are not
405 included in our control group. Our choice to include seropositive controls allows us to examine the role
406 of *IL2* variants in the stages of disease progression beyond the initial mounting of an immune response
407 to an HPV infection. Since the motivation for this study was to investigate a potential mechanism for

408 current cigarette smoking, these controls allow us to focus on the later stages of disease progression
409 where current cigarette smoking is most likely relevant. Unfortunately, we do not have cancer-free
410 individuals with persistent genital HPV-infection defined by HPV DNA status in our study, therefore
411 we cannot separate our inferences regarding the joint effect of *IL2* variants and cigarette smoking, or
412 *IL2* variants alone, on HPV persistence and tumor progression.

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

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

431 IL-2 is central to T lymphocyte immune response, but by no means is it the only influential
432 cytokine or immune factor to potentially modulate the effect of cigarette smoking in cervical or vulvar
433 cancer risk. For example, cervical cancer risk is reduced among carriers of the HLA Class II
434 DRB1*13/DBQ1*0603 alleles (62), and possibly certain polymorphisms in genes coding for interferon
435 gamma (63) and interleukin 10 (64). The possibility that these polymorphisms, or polymorphisms of
436 other cytokines, receptors, or immune factors, modify the association between cigarette smoking and
437 cancer risk has yet to be explored.

438 Substantial progress in recent years towards development and uptake of prophylactic HPV
439 vaccines provides hope for reducing the burden of HPV infection and associated neoplasms in the
440 future (65). Nonetheless, there remain a large number of women that will not benefit from the vaccine
441 as they have already acquired HPV infection, are beyond the target age of vaccination, or live in low-
442 resource regions of the world that are challenged by the high cost and distribution of a vaccine (66,
443 67). Identification of gene-environment interactions that contribute to cervical and vulvar cancer risk
444 may help shed light on the biological mechanisms leading to cancer, and potentially identify women
445 who are at increased risks of developing these malignancies.

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Table 1. Selected characteristics of cervical and vulvar squamous cell carcinoma cases

	Cervical cancer cases (N=399)	Vulvar cancer cases (N=486)
Mean age at diagnosis (years)	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
Number 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 months	31.4	29.6

6 to 59 months	31.4	32.3
≥5 years	37.2	38.1
First degree relative with anogenital cancer (%)		
Yes	3.7	8.0
No	96.3	92.0

* Represents the percentage of tested individuals

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 control
						OR (95% CI)	<i>p</i> value**	OR (95% CI)
rs2069762	495	5' flanking	T/G	0.23	23%	1.19 (0.69-2.06)	0.68	1.30 (0.82-2.04)
rs2069763	993	Exon 1	G/T	0.38	23%	1.11 (0.66-1.85)	0.48	1.08 (0.69-1.67)
rs2069777	2038	Intron 1	C/T	0.07	21%	1.23 (0.45-3.37)	0.69	0.90 (0.34-2.33)
rs2069778	2340	Intron 1	C/T	0.18	18%	0.72 (0.35-1.46)	0.54	0.60 (0.31-1.11)

*rs number refers to the National Center for Bioinformatics (NCBI) dbSNP build 127 reference sequence number.

†Locations are with respect to the first nucleotide position in the NCBI 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

||OR, odds ratio; CI, confidence interval. 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 *IL2* tagSNP genotypes and cigarette smoking among controls.

**Likelihood ratio test *p* value (39)

Table 3. Interaction odds ratios 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	Non-smokers		Smokers	Non-smokers	
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 (NCBI) dbSNP build 127 reference sequence number.

†IOR, interaction odds ratio; CI, confidence interval.

‡Dominant genetic model.

§Recessive genetic model.

Table 4. Interaction odds ratios 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	Non-smokers		Smokers	Non-smokers	
<u>T</u> <u>T</u> CC	0.37	0.33	1.00	0.32	0.32	1.00
<u>G</u> CC	0.28	0.28	0.93 (0.63-1.36)	0.32	0.25	1.34 (0.94-1.92)
T <u>G</u> <u>C</u> T	0.15	0.18	0.81 (0.52-1.26)	0.18	0.16	1.16 (0.79-1.71)
TGCC	0.13	0.14	0.89 (0.57-1.41)	0.11	0.16	0.71 (0.47-1.07)
T <u>G</u> <u>T</u> 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.

†IOR, interaction odds ratio; CI, confidence interval. Calculated assuming a log-additive genetic model.

Table 5. Interaction odds ratios between *IL2* 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	Non-smokers		Smokers	Non-smokers	
T <u>T</u> CC / <u>G</u> GCC	0.18	0.22	1.00	0.25	0.20	1.00
T <u>T</u> CC / T <u>T</u> CC	0.17	0.10	2.08 (1.01-4.30)	0.08	0.08	0.85 (0.41-1.78)
T <u>T</u> CC / T <u>G</u> C <u>T</u>	0.08	0.12	0.83 (0.37-1.87)	0.11	0.11	0.82 (0.43-1.56)
T <u>T</u> CC / T <u>G</u> CC	0.11	0.08	1.69 (0.76-3.79)	0.07	0.08	0.74 (0.36-1.51)
T <u>G</u> C <u>T</u> / <u>G</u> GCC	0.08	0.09	1.09 (0.47-2.53)	0.13	0.05	2.09 (0.98-4.46)
<u>G</u> GCC / <u>G</u> GCC	0.09	0.07	1.68 (0.72-3.95)	0.07	0.06	0.97 (0.44-2.13)
T <u>G</u> CC / <u>G</u> GCC	0.05	0.06	1.05 (0.41-2.72)	0.05	0.08	0.55 (0.26-1.17)
T <u>T</u> CC / T <u>G</u> <u>T</u> C	0.03	0.05	0.72 (0.22-2.34)	0.04	0.09	0.37 (0.16-0.85)
T <u>G</u> <u>T</u> C / <u>G</u> GCC	0.06	0.04	1.70 (0.63-4.58)	0.05	0.03	1.25 (0.47-3.33)
T <u>G</u> C <u>T</u> / T <u>G</u> CC	0.03	0.06	0.65 (0.20-2.08)	0.03	0.08	0.37 (0.15-0.87)
T <u>G</u> C <u>T</u> / T <u>G</u> C <u>T</u>	0.04	0.03	1.66 (0.54-5.11)	0.02	0.02	0.95 (0.26-3.47)
T <u>G</u> C <u>T</u> / T <u>G</u> <u>T</u> C	0.03	0.02	2.28 (0.55-9.37)	0.03	0.04	0.53 (0.19-1.45)
T <u>G</u> CC / T <u>G</u> CC	0.02	0.02	1.65 (0.38-7.19)	0.02	0.02	0.72 (0.20-2.55)
T <u>G</u> CC / T <u>G</u> <u>T</u> C	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.

†IOR, interaction odds ratio; CI, confidence interval.

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 (NCBI) dbSNP build 127 reference sequence number.

†OR, odds ratio; CI, confidence interval. All controls were seropositive for HPV16 or HPV18 L1 antibodies.

Supplementary Table 1. Assay primer and probe sequences for *IL2* tagSNPs*

TagSNP†	rs2069763	rs2069777	rs2069778
Forward primer sequence	5'-TGCACCTACTTCAAGTTCTACAAAGAA-3'	5'-CATCCAAGCTCCTAGGCTACATTAG-3'	5'-GCTGTTTTCTGAAGAAAATTTCTCCACAT-3'
Reverse primer sequence	5'-AAAGGAAATATACTTACATTAATTCCATTCAAAATCATCTG-3'	5'-TGGCACCAGATTTTGTTCATTCTCT-3'	5'-GCGCTTTCAATTCACCACTACAA-3'
Probe sequence 1‡	5'-ATCCAGCAGTAAATG-3'	5'-ACTGGCACAGCTACTA-3'	5'-TTCTACAAATTCGGGTTTAA-3'
Probe sequence 2§	5'-TAAATCCAGAAGTAAATG-3'	5'-ACTGGCACAACACTACTA-3'	5'-ATTCTACAAATTCAGGTTTAA-3'
Orientation	Forward	Forward	Forward

*The rs2069762 was genotyped using Applied Biosystems Pre-Designed Taqman® genotyping assay for which the probes and primer sequences are proprietary information.

†Rs number refers to the National Center for Bioinformatics (NCBI) dbSNP build 127 reference sequence number.

‡Probe sequences were labeled with a 5' reporter VIC dye and 3' minor groove binding non-fluorescent quencher.

§Probe sequences were labeled with a 5' reporter FAM dye and 3' minor groove binding non-fluorescent quencher.