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Genetic variants of a BH3-only pro-apoptotic gene, *PUMA*, and risk of HPV16-associated squamous cell carcinoma of the head and neck

Ziyuan Zhou¹, Erich M. Sturgis^{1,2}, Zhensheng Liu¹, Li-E Wang¹, Qingyi Wei¹, and Guojun Li^{1,2,*}

¹Department of Epidemiology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

²Department of Head and Neck Surgery, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

Abstract

P53 up-regulated modulator of apoptosis (PUMA) is a critical factor in the intrinsic apoptotic pathway. Through PUMA-dependent mechanisms, human papillomavirus 16 (HPV16) oncoprotein may affect apoptosis by E6-mediated p53 degradation. To examine whether the *PUMA* variants modify the association between HPV16 serology and risk of squamous cell carcinoma of the head and neck (SCCHN), we genotyped two polymorphisms in the *PUMA* promoter (rs3810294 and rs2032809) in 380 cases and 335 cancer-free controls of non-Hispanic whites, who were frequency-matched by age (± 5 years), sex, smoking and drinking status. We found that each individual polymorphism had only a modest impact on risk of SCCHN, particularly in oropharyngeal cancer for rs3810294 and non-oropharyngeal cancer for rs2032809. After we stratified the individuals by HPV16 serology, and used those with the corresponding common homozygous genotype and HPV16 seronegativity as the reference group, for each polymorphism we found that the risk of SCCHN associated with HPV16 seropositivity was higher among those with variant genotypes than those with the corresponding common homozygous genotype. Notably, this effect modification was particularly pronounced in several subgroups including never smokers, never drinkers, younger patients, and patients with oropharyngeal cancer. Furthermore, we also characterized the functional relevance of the two polymorphisms to explore the genotype-phenotype correlation. Our results suggested that the *PUMA* promoter polymorphisms may be a biomarker for risk of HPV16-associated SCCHN, particularly in never smokers, never drinkers, younger patients, and patients with oropharyngeal cancer. Larger studies are needed to validate our findings.

Keywords

PUMA polymorphisms; HPV16; genetic susceptibility; molecular epidemiology; squamous cell carcinoma of the head and neck

*Corresponding Author: Guojun Li, Department of Head and Neck Surgery, Unit 1445, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Tel: 713 792 0227; Fax: 713 794 4662; gli@mdanderson.org.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is the eighth most common cancer worldwide and has an estimated incidence of 49,260 new cases and 11,480 deaths in the United States in 2010 [1]. In addition to the well established risk factors of smoking and alcohol use, epidemiological evidence suggests that human papillomavirus (HPV) is a major contributor to the risk of SCCHN, particularly squamous cell carcinoma of oropharynx (SCCOP) [2,3]. Two large studies have shown that the overall HPV prevalence in head and neck cancer is approximately 22%~26%, of which there are nearly 20 high-risk HPV types, while type 16 accounting for approximately 90% of HPV positive specimens [4,5]. Although infection with oncogenic types of HPV is very common, HPV-associated cancer is a rare outcome among the HPV-infected population, implying that except for HPV infection, the host's genetic predisposition may contribute to inter-individual variation in susceptibility to HPV-associated SCCHN in the general population. Our previous studies have indicated that genetic variants in genes controlling cell cycle check-point, apoptosis or DNA repair pathways may play an essential role in carcinogenesis of SCCHN [6–9].

Deregulated apoptosis in the extrinsic or intrinsic pathway, which usually can be triggered upon both genotoxic and non-genotoxic cellular stresses, is a leading characteristic of development of human cancers including SCCHN. In the intrinsic (mitochondrial) pathway, a cell could die (apoptosis) or survive largely upon interactions among Bcl-2 family proteins [10]. As a member of the pro-apoptotic Bcl-2 homology 3 (BH3) subfamily of Bcl-2 family, P53 up-regulated modulator of apoptosis (PUMA) is identified as one potent cell killer. Under genotoxic stress, PUMA can be rapidly induced by p53 in response to stimuli and may account for virtually all of the pro-apoptotic activity of p53 [11,12]. Both *in vivo* and *in vitro* studies indicated that deficiency in PUMA may result in protection from apoptosis induced by genotoxic agents or γ -irradiation [13,14]. In addition to p53-dependent manner, *PUMA* can also respond to other transcription factors in a p53-independent manner under non-genotoxic stress. Although a recent study implies that adenoviral gene delivery of *PUMA* induced apoptosis and chemosensitization more potently than did adenoviral delivery of p53 in SCCHN cells [15], an earlier study indicated that the *PUMA*-deficient mice did not show increased risk for spontaneous malignancies [12]. However, recent animal studies demonstrate a dual role of *PUMA*-deficiency in tumorigenesis [16,17]. Loss of *PUMA* can not only accelerate B-cell lymphomagenesis but also result in higher leukemic burden and increase formation of intestinal tumors and adenoma [18]. Moreover, PUMA-mediated apoptosis might contribute to carcinogen-induced hepatocarcinogenesis via compensatory proliferation [17]. Thus, the PUMA-mediated apoptosis suppresses tumorigenesis, perhaps through both p53- and non-p53-dependent mechanisms. For example, the induction of PUMA by genotoxic stress or other classes of agents is strictly p53 dependent and is abolished in p53-deficient human cancer cells [11, 12], while the PUMA induction in response to DNA damage is also p53-independent. Such DNA damage activates ATM or ATR, and this activates CHK1 or CHK2. CHK1 and CHK2 activate E2F1 and the transcription factor E2F1 transcribes the p73 protein, which mediate apoptosis through the transcription of PUMA, NOXA and BAX [11, 12]. These factors can regulate the expression of *PUMA* by directly binding to the response elements in the promoter region [11,12] (Fig. 1).

Recent studies have shown association of high risk HPV infection, mainly the HPV16, with increased SCCOP risk [4,5,19–22]. HPV is primarily attributed to malignant transformation by their oncoproteins E6 and E7. HPV E6 can induce p53 degradation, which may result in the disabling PUMA-mediated apoptosis through the p53-dependent pathway. Furthermore, by binding to the inactivated pRb, HPV E7 can cause ectopic E2F expression and trigger defective apoptosis and cell cycle progression, perhaps partly through the p53-independent

E2F-PUMA interaction pathway [23,24]. Several studies also demonstrated that the expression of p53 downstream targets such as PUMA and p21, by preventing the E6-mediated p53 degradation, can be activated in a p53-dependent manner in HPV positive human cervical HeLa and SiHa cells, followed by enhanced cell cycle arrest and apoptosis [25,26]. Therefore, PUMA may affect regulation of apoptosis, which subsequently may influence risk of human cancers including SCCHN. To date, no published studies have investigated the association between *PUMA* polymorphisms and the risk of HPV-associated SCCHN. In this study, we hypothesized that functional polymorphisms in the *PUMA* promoter may modify the risk of SCCHN in particular those of the oropharynx associated with HPV16 seropositivity. To test this hypothesis, we evaluated the joint effect between the *PUMA* promoter polymorphisms and HPV16 seropositivity on risk of SCCHN in a case-control study of 380 cases and 335 controls.

Materials and Methods

Patients and control samples

Details of this SCCHN case-control study population were previously described elsewhere [7]. Briefly, all histopathologically confirmed incident SCCHN patients were recruited consecutively as part of an ongoing molecular epidemiology study of SCCHN at the Head and Neck Surgery Clinic at The University of Texas M.D. Anderson Cancer Center between May 1996 and May 2002. The response rate of eligible patients who signed an informed agreement for participating in the study was approximately 95%. Excluded patients included those with second primary tumors; primary tumors of the sinonasal tract and nasopharynx; primary tumors outside the upper aerodigestive tract; cervical metastases of unknown origin; and histopathologic diagnoses of tumors other than squamous cell carcinoma, as well as patients with known immune symptoms or who had received recent blood transfusions within the last 6 months or who were receiving immunosuppressive therapy. Finally, 380 non-Hispanic white SCCHN patients were included in this study.

Controls were recruited from a pool of cancer-free subjects including attendees of the Kelsey-Seybold Foundation (a multispecialty physician practice with multiple clinics throughout the Houston metropolitan area) and cancer-free visitors who accompanied cancer patients to outpatient clinics at M.D. Anderson Cancer Center but who were genetically unrelated to the SCCHN patients. In this cancer-free control pool, a short questionnaire was used to determine each individual's willingness to participate in the study before he or she was interviewed and asked to provide demographic and epidemiologic information including age, sex, ethnicity, smoking history, and alcohol consumption. Exclusion criteria for the control group included having had cancer previously, having known immune symptom or received blood transfusions within the last 6 months, or receiving immunosuppressive therapy. Approximately 78% of eligible subjects responded to the survey. As a result, 335 cancer-free control individuals were selected from the pool of potential controls by frequency matching on age (± 5 years), gender, ethnicity, and smoking and alcohol drinking status. These variables were further adjusted for in later multivariable logistic regression analyses to control for any residual confounding effect.

"Ever smokers" were those who had smoked more than 100 cigarettes in their lifetime, and the rest were "never smokers"; "ever drinkers" were those who drank alcoholic beverages at least once a week lasting for more than one year, and the rest were "never drinkers". After a written informed consent was given, each individual provided a one-time 30-ml blood sample collected in heparinized tubes. The research protocol was approved by both the MD Anderson Cancer Center and Kelsey-Seybold Institutional Review Boards.

HPV16 serologic testing

According to a previously described standard enzyme-linked immunoadsorption assay [27], antibody against the HPV16 L1 capsid protein in the plasma of study participants was tested by using HPV16 L1 virus-like particles generated from recombinant baculovirus-infected insect cells. A standard pooled serum known to be at the cutoff point for HPV16 L1 seropositivity in a previous study was used in the current study to determine the HPV16 L1 seropositivity cutoff level [27]. Two more tests were performed in the samples within 15% of the cutoff point, and only those defined as positive in all three tests were considered as positive. Plasma samples were treated with heparinase I before testing to avoid the heparin binding interference. We did not detect discernible reaction-differences between the serum samples and the heparinized plasma samples obtained from three individuals. For quality control, 10% of the samples were randomly chosen for retesting and 100% concordance was observed in the repeat tests.

Selection and genotyping of candidate SNPs

The NCBI dbSNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP>) and online bioinformatics tool FuncPred (<http://manticore.niehs.nih.gov/snpfunc>) were used to identify and predict potentially functional SNPs in the *PUMA* gene. We searched for the *PUMA* gene within a nearly 14.4 kb region in chromosome 19 (from 52,415,560 bp to 52,429,386 bp) among Caucasian populations with European ancestry (CEPH: Utah residents with ancestry from northern and western Europe). We first scanned all reported common SNPs (minor allele frequency, MAF>0.05); then evaluated potential functions for SNPs that located in either the coding region or the 5'-untranslated region known as transcriptional regulatory region in the upstream of the start codon (from -2000 to +1), as well as for SNPs located in 3'-UTR in the downstream of the coding region (+1kb downstream) for potential miRNA binding sites. We further used the public HapMap SNP database (<http://www.hapmap.org/>) to assess the linkage disequilibrium (LD) values between pro-target SNPs. As a consequence, of the 67 SNPs reported, neither common non-synonymous SNPs located in the coding region nor common SNPs located in the 3'-UTR that may potentially cause the change of miRNA-binding were found, but there were two common SNPs located in the promoter region (rs2032809 in 5'-UTR and rs3810294 in intron 1) that fitted the criteria of SNPs selection.

Genomic DNA obtained from the buffy coat of whole blood samples was extracted by the Qiagen DNA blood mini kit (Valencia, CA). For PCR amplification, we designed the following primers with mismatched base pairs to generate a new restriction site and to amplify the target fragments in 5'-UTR region (rs2032809) and in intron1 (rs3810294) region of *PUMA*: 5'-GAATAATCGGGGAAAGCGAAAGAAG-3' (forward, the bold and underlined base is mismatched) and 5'-AGTGTGGGGCTGGCTGAGTAAG-3' (reverse) for rs2032809; 5'-AGGAGGAGCAGGTCAGCAGGGA-3' (forward) and 5'-TGCCCATCACCGTATGCACGGG-3' (reverse) for rs3810294; the 191bp- and 199bp-PCR products of these primers were digested by *Mbo*II and *Tsp45*I restriction enzymes (New England Biolabs, Beverly, MA), respectively, to identify the genotypes of rs2032809 G>A and rs3810294 C>T polymorphic loci. As quality control, approximately 10% of the DNA samples were randomly selected for re-genotyping and 100% concordance was observed in repeat tests.

Quantitative measurement of *PUMA* mRNA expression

Total RNA was extracted from phytohemagglutinin-stimulated peripheral blood mononuclear cells among 91 cancer-free controls, and the concentration of RNA and cDNA was determined by using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The mRNA expression levels of *PUMA* were determined by

quantitative real-time reverse transcriptase-PCR (qRT-PCR) method, and normalized with the 18S fraction of ribosomal RNA expression as internal reference. The qRT-PCR reaction and expression levels of *PUMA* and 18S mRNA were analyzed by using the ABI 7900HT Sequence Detection (Applied Biosystems, Foster City, CA). *PUMA* and 18S mRNA levels were separately amplified in duplicates, and the expression level of *PUMA* relative to that of 18S was calculated according to the derived cycle threshold values (Ct) by using the equation $\text{ratio} = \text{Ct}_{PUMA}/\text{Ct}_{18S} \times 100\%$.

Electrophoretic mobility shift assay (EMSA)

The nuclear extracts from MDA866 head and neck cancer cells were prepared according to the method of Andrews and Faller [28]. Complementary single-stranded oligonucleotides (5'-ATGCGGAGGGGGTGACGGCCCCACAGAGACA-3' for the C allele and 5'-ATGCGGAGGGGGTGATGGCCCCACAGAGACA-3' for the T allele of rs3810294; 5'-GGAAAGCGAAAGAGGGGGGAAAGTGAAAGAG-3' for the G allele and 5'-GGAAAGCGAAAGAGGAGGGAAAGTGAAAGAG-3' for the A allele of rs2032809) were biotin-labeled using the 3'-end biotin labeling kit (Thermo Scientific, Rockford, IL) and re-annealed to perform the DNA binding assays using the LightShift Chemiluminescent EMSA kit (Thermo Scientific, Rockford, IL) according to manufacturer's experimental procedures. The competition was performed with a 50-fold excess of unlabeled oligonucleotides.

Statistical analysis

The differences in distributions of selected variables between the patients and controls, including HPV16 serological status and *PUMA* genotypes, were examined by using the χ^2 test. Odds ratios (OR) and their 95% confidence intervals (95%CI) were calculated by using both univariate and multivariable logistic regression models for evaluating the association of *PUMA* genotypes and HPV16 seropositivity with the risk of SCCHN. Moreover, we evaluated the joint effects of *PUMA* genotypes and HPV16 seropositivity on risk of SCCHN, which were further stratified by tumor sites, age, smoking and drinking status. We use the *t* test to compare the expression levels of *PUMA* between different genotypes of the two polymorphisms. All of the statistical analyses were performed by using Statistical Analysis System software (Version 9.1; SAS Institute, Cary, NC), with two-side tests and a significance level of $P < 0.05$.

Results

Demographic and risk factors for study subjects

The demographic characteristics and risk factors of this study population are summarized in Table 1. The cases and controls appeared to be adequately frequency-matched for sex, age, smoking status, and alcohol use. However, we found that cases were much more likely than controls to be HPV16 seropositive ($P < 0.0001$) and HPV16 seropositivity was associated with significantly increased risk of SCCOP (adjusted OR=5.7; 95%CI, 3.6–8.9), but not for SCCHN at non-oropharyngeal sites (adjusted OR=0.7; 95%CI, 0.4–1.3).

Association of *PUMA* variants with the risk of SCCHN

The distribution of *PUMA* genotypes and association with risk of SCCHN are shown in Table 2. We originally recruited 380 eligible cases and 335 controls in this study, and 10 cases and 17 controls failed in the genotyping assays. Thus, 370 cases and 318 controls were included in the final genotyping analysis. The frequencies of both SNPs among controls were in agreement with Hardy-Weinberg equilibrium ($P = 0.437$ for rs3810294, $P = 0.231$ for rs2032809). For each polymorphism, because of the relatively small number of

homozygous minor genotype, we therefore combined the homozygous minor genotype and heterozygous genotype for the comparison. We found that the combined *PUMA* rs3810294 CT+TT genotypes were associated with a significantly increased SCCHN risk (OR=1.5, 95% CI, 1.0–2.1), compared with the CC genotype; and the combined *PUMA* rs2032809 GA+AA genotypes were also associated with a significantly increased SCCHN risk (OR=1.5, 95% CI: 1.1–2.0), compared with the GG genotype. Such risk was especially higher for patients with SCCOP for *PUMA* rs3810294 (OR=1.7, 95% CI, 1.1–2.7) and for patients with SCCHN at non-oro-pharyngeal sites for *PUMA* rs2032809 (OR=1.6, 95% CI, 1.1–2.4). Both SNPs showed a significant trend of the increasing SCCHN risk with increasing numbers of variant alleles ($P_{\text{trend}} = 0.027$ for rs3810294 and $P_{\text{trend}} < 0.001$ for rs2032809). However, SNP rs3810294 only exhibited the allele-dose effects on risk of tumors at HPV-related sites (SCCOP, $P_{\text{trend}} = 0.026$) but not at HPV-unrelated sites (non-oro-pharynx, $P_{\text{trend}} = 0.134$), while SNP rs2032809 exhibited allele-dose effects on risk of tumors at both oro-pharynx ($P_{\text{trend}} = 0.03$) and non-oro-pharynx ($P_{\text{trend}} = 0.001$) (Table 2).

Joint effects of *PUMA* variants and HPV16 seropositivity on the risk of SCCHN

As summarized in Table 3, we combined the HPV16 seropositivity and *PUMA* genotypes to estimate their joint effects on SCCHN risk. Compared with the group of rs3810294 CC genotype and HPV16 seronegativity, the group of the CT/TT genotypes and HPV16 seronegativity had an increased SCCHN risk (OR, 1.3, 95% CI, 0.9–2.0), the group of the CC genotype with HPV16 seropositivity had an OR of 2.5 (95% CI, 1.6–4.0), and the group of both CT/TT genotypes and HPV16 seropositivity had the highest OR of 6.5 (95% CI, 2.6–16.2). Similarly, compared with those with the rs2032809 GG genotype and HPV16 seronegativity, the group of the GA/AA genotypes and HPV16 seronegativity had significantly increased SCCHN risk (OR=1.5; 95% CI, 1.0–2.1), the group of GG genotype and HPV16 seropositivity had an OR of 2.8 (95% CI: 1.4–5.4), and the group of both GA/AA genotypes and HPV16 seropositivity had the highest OR of 4.3 (95% CI, 2.4–7.4) (Table 3). Moreover, when the cases were stratified by sites, we also found that such effect modification of both polymorphisms on risk of HPV-associated cancers was evident for SCCOP but not for SCCHN at non-oro-pharyngeal sites. This modification effect might suggest an interaction between HPV16 seropositivity and *PUMA* variants in the etiology of SCCHN, especially for SCCOP, but larger studies are needed to verify these findings.

Stratification of the joint effects of *PUMA* variants and HPV16 seropositivity on SCCOP risk

As we found that the modifying effects of *PUMA* variants on risk associated with HPV16 seropositivity was particularly evident for SCCOP, we stratified the effect modification on the association between *PUMA* variants and HPV16 serology on risk of SCCOP by smoking/drinking status and age (Table 4). For each polymorphism, we observed that the joint effects of *PUMA* genotypes and HPV seropositivity on risk of oro-pharyngeal cancer was much stronger in never smokers than in ever smokers and in never drinkers than in ever drinkers. In addition, when we stratified the joint effect of *PUMA* genotypes and HPV16 seropositivity on risk of SCCOP by age (mean age of controls), we found that the modifying effects of *PUMA* variants on SCCOP risk associated with HPV16 seropositivity was greater among young subjects (aged ≤ 58 year) than among older subjects (aged > 58 year).

Genotype-phenotype correlation analysis of *PUMA* polymorphisms

To explore the functional relevance of the polymorphisms in *PUMA* promoter, we used the qRT-PCR method to determine *PUMA* mRNA expression levels in peripheral lymphocytes among 91 cancer-free individuals for the genotype-phenotype correlation analysis between different genotypes of the two polymorphisms. In this analysis, rs3810294 CC genotype was associated with a statistically higher expression level than CT/TT genotypes ($P = 0.003$, Fig.

2); rs2032809 GG genotype was associated with a lower expression level than GA/AA genotypes but the difference was not significant ($P = 0.636$, Fig. 2). To determine the potentially differential regulation of the *PUMA* promoter activity, we further performed the EMSA to verify whether these two polymorphisms may change the binding affinity of any transcriptional factor to the *PUMA* promoter. For both polymorphisms, we found that the C allele (for rs3810294) and A allele (for rs2032809) had a significantly stronger binding to the nuclear proteins extract than T and G allele, respectively, indicating a potentially different binding affinity of nuclear transcription factors to the two alleles of each polymorphism (Fig. 3). These results might only suggest a potentially functional correlation between the two promoter SNPs and *PUMA* expression and might not indicate the exact changes of *PUMA* expression (down- or up-regulation) (Fig. 3). Therefore, further functional studies for the two SNPs are needed to elucidate how and to what extent the two polymorphisms in *PUMA* promoter may affect *PUMA* expression.

Discussion

In this study, we found that both functional SNPs in the regulating region of *PUMA* appeared to have a moderate effect on overall risk of SCCHN but significantly contributed to the risk of HPV16-associated SCCOP, particularly in never smokers, never drinkers, and young subjects (aged ≤ 58 year). Such effect modification might suggest a potential interaction between *PUMA* polymorphisms and the HPV16 infection on risk of SCCHN. However, because we did not have enough study power to detect the significance for interaction given the small sample size in each stratum, future studies with large sample sizes are needed to verify our results.

As a protection against carcinogenesis, apoptosis is induced by a variety of stimuli including genotoxic and non-genotoxic stresses. As one of the Bcl-2 family members, PUMA is a critical mediator of apoptosis, and its pro-apoptotic activity is transcriptionally activated by p53 in response to genotoxic stresses, such as DNA damage, or by other transcription factors independent of p53 in response to non-genotoxic stresses, such as growth factors and cytokines withdrawal [12,29]. The expression of *PUMA* is normally maintained at a very low level, and the activity of PUMA seems to be exclusively transcription-regulated. Once intolerable and unrecoverable damages happened to a cell, this would rapidly induce PUMA, interacting with the multi-domain Bcl-2 family members, transducing death signals to the mitochondria and then inducing mitochondria dysfunction, cytochrome c release, caspase-3 and caspase-9 activation, thereby triggering cell apoptosis [30–32]. Studies of *PUMA*-knockout cell models or animal models indicate that deficiency or inhibition of PUMA results in high protection from p53-mediated or non-p53-mediated apoptosis induced by γ -irradiation or cytokine withdraw. In contrast, over expression of *PUMA* results in rapid and complete apoptotic responses regardless of p53 genotypes in a variety of cancer cell lines [31–35], leading to growth-suppression of different types of tumors *in vivo* [15,32,36]. Although the role of PUMA in apoptosis and tumorigenesis remains to be fully understood, one possible mechanism is that several transcription factors, such as p53 or E2F, can alter *PUMA* expression through their respective binding sites in the regulating regions of the *PUMA* gene [11,12], thereby prompting or blocking apoptosis induction, and accordingly altering susceptibility to cancers, including SCCHN. Consistent with such a hypothesis, we found in the present study that, for each of the two functional SNPs in the promoter, respective *PUMA* genotypes containing the minor allele were individually associated with a moderately increased risk of SCCHN after adjustment for multivariable factors, including age, sex, smoking and drinking status (OR_{CT+TT} for rs3810294 and OR_{GA+AA} for rs2032809 both are 1.5). Until now, there is no epidemiologic study that has evaluated the association between *PUMA* polymorphisms and risk of cancers, but our findings in this study are indirectly consistent with results of a few very recent studies *in vivo*, which

demonstrated that high expression of *PUMA* could efficiently suppress the growth of SCCHN or increase SCCHN's sensitivity to chemotherapeutic agents [32,34]; meanwhile, inhibition of *PUMA* expression was associated with increased chemoresistance of SCCHN cells and prolonged survival of cancer-bearing mice [15,32].

We further performed genotype-phenotype tests to verify whether these two polymorphisms may change the *PUMA* expression. We found that the rs3810294 T allele, compared to the C allele, had significantly reduced binding affinity to nuclear transcription factors (Fig. 3A) and was associated with significantly reduced *PUMA* expression level (Fig. 2), while compared to the A allele, the rs2032809 G allele had significantly reduced binding affinity (Fig. 3B) and was associated with non-significantly reduced expression level (Fig. 2). Although the functional relevance of these two SNPs in promoter has not yet been elucidated, our results might suggest a functional correlation between the two SNPs and *PUMA* expression, which may provide preliminary evidence of biological plausibility for the observed association in current study. However, our results from the EMSA assays for binding affinities to nuclear proteins extract are non-specific, and such results only suggest that these genetic variants may alter *PUMA* expression (either up- or down-regulation) and subsequently alter cancer risk. Therefore, identification of specific transcription factors binding to the nearby regions and understanding of the exact mechanism by which these two polymorphisms affect *PUMA* expression warrant further *in vitro* and *in vivo* studies.

In the recent decades, significantly increasing incidence rates have been seen for HPV-related SCCHN, especially for oropharyngeal cancer [3,4,22,37]. HPV16 viral oncoproteins E6 and E7 can degrade the p53 and pRb-E2F complex, respectively, disrupt the p53-related or E2F-related pathway functions, and thus contribute to the carcinogenesis including SCCHN [23,24]. As an essential mediator of apoptotic pathways, PUMA can be activated by the direct binding of multiple transcriptional factors including p53 and E2F [11,12]. Although there were no *in vivo* proofs that the HPV16 infection can directly regulate the *PUMA* expression, the findings of *in vitro* studies have revealed that HPV16 oncoprotein E6 can stimulate PUMA transcription in a p53-dependent manner in multiple cancer cells by targeted inhibition of HPV16 oncoprotein E6 expression [38]. This PUMA transcription can also be stimulated in HPV-positive SiHa cells by activating p53 expression through enforcedly expressing a potent E6 antagonist to prevent E6-mediated p53 degradation, and consequently being reverted to the apoptotic phenotype [26]. In another recent study, by inhibiting E6-mediated p53 degradation to rescue its tumor suppressor function, a small-molecule RITA (reactivation of p53 and induction of tumor cell apoptosis) can activate the transcription of p53 targets including PUMA. Subsequently, such activation results in p53-dependent apoptosis and cell cycle arrest in multiple cancer cells containing HPV16 and substantial suppression of cervical carcinoma xenografts *in vivo* [25]. Therefore, HPV16 may work synergistically with PUMA in blocking apoptosis and facilitating the development of cancers, at least partly, through the HPV-p53-PUMA proapoptosis axis, which suggests an expectation of further impaired apoptosis and increased tumorigenesis as well. In agreement with this scenario, we observed in the current study that *PUMA* polymorphisms and HPV16 seropositivity may have potentially joint effects on increased risk of SCCOP. Although we did not have adequate study power to detect this interaction due to the limit sample size in this study, the trends of effect modification suggested a potential interaction between the *PUMA* variants and HPV16 seropositivity.

In this study, we did find that the modifying effects of *PUMA* variants on the risk associated with HPV16 seropositivity were more pronounced for oropharyngeal as opposed to non-oropharyngeal cancers, and the modifying effects were even greater in never smokers than in ever smokers and in never drinkers than in ever drinkers. Such results are consistent with other's and our previously published studies, in which HPV16 and genetic polymorphisms

may have interactive effects on risk of SCCOP, particularly in never smokers and never drinkers, because HPV-associated SCCOP patients are more likely to be never smokers and never drinkers [7,39]. Our observations of more pronounced modifying effects in younger subjects than in older subjects might be partly explained by the increased oral HPV16 prevalence in young adults perhaps owing to distinct changing in sexual behaviors or that susceptible groups develop cancers at a younger age [37,40]. So far, only a few studies *in vivo* have examined the possible role of PUMA in tumor growth, chemoresistance or survival anticipation [15,32,34], but no population study has reported the association between the *PUMA* polymorphisms and the risk of cancers including SCCHN. To the best of our knowledge, therefore, the current study is the first to report the association of *PUMA* polymorphisms with risk of SCCHN as well as their effect modification on SCCHN risk associated with HPV16 seropositivity along with smoking and drinking.

There are several limitations for our present study. First of all, the HPV16 serology tests can only indicate whether the study subjects had previous HPV16 exposure but could not specify the anatomic location or time of viral exposure. Therefore, with this uncertainty applied to both the cases and controls, possible false-negative HPV16 cases might result in misclassification of HPV16 status. Thus, we will carefully verify the tumor HPV status (such as p16 immunohistochemical staining) and assess its role in the development of SCCHN, particularly in oropharyngeal cancer, in our future studies when a much larger patient cohort with HPV-associated tumor becomes available.

However, several studies have reported a reasonable concordance between HPV16 seropositivity (47%~52% for HPV16 L1 antibodies) and HPV16 DNA positivity in tumor tissues [19,20,41]. More recently, a large study reported a high correlation between HPV DNA and HPV serology tests [42]. More importantly, the use of serologic status allowed for the inclusion of a cancer-free control group. Another limitation in this study arose from the sample size, because we did not have enough study power for stratified analysis or interaction analysis. Thus, there is a possibility that our results could be by chance. Additionally, we can not deduce similar conclusions with certainty to other ethnic populations, because our study included only non-Hispanic white participants.

In conclusion, our results suggest that *PUMA* polymorphisms have a moderate effect on risk of SCCHN but might have a joint effect with HPV16 seropositivity on risk of SCCHN, particularly among never smokers, never drinkers, younger patients, and patients with SCCOP. Additionally, because HPV oncoproteins may potentially interact with many other genes involved in the p53-mediated apoptosis pathway, further studies are needed to elucidate the mechanisms underlying the interactions among PUMA, HPV16 and other genes in the p53-dependent or p53-independent pathways in carcinogenesis of SCCHN.

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Abbreviations

CI confidence interval

HPV	human papillomavirus
OR	odds ratio
PCR	polymerase chain reaction
SCCHN	squamous cell carcinoma of the head and neck
SCCOP	squamous cell carcinoma of the oropharynx

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Figure 1.

Figure 2.

Figure 3.

Table 1
Frequency distribution of demographic and risk factors in SCCHN patients and controls

Characteristics	Patients (n=380)		Controls ^a (n=335)		P value ^b
	No.	%	No.	%	
Age (years)					0.526
< 40	32	8.4	27	8.1	
41 – 55	142	37.4	109	32.5	
56 – 70	159	41.8	157	46.9	
> 70	47	12.4	42	12.5	
Sex					0.091
Male	285	75.0	269	80.3	
Female	95	25.0	66	19.7	
Ethnicity					
Non-Hispanic white	380	100.0	335	100.0	
Tobacco smoking					0.588
Ever	278	73.2	239	71.3	
Never	102	26.8	96	28.7	
Alcohol drinking					
Ever	296	77.9	240	71.6	0.054
Never	84	22.1	95	28.4	
Tumor site					
Oropharynx	187	49.2			
Non-Oropharynx	193	50.8			

^aThe controls were selected by frequency matching to the patients on the factors shown in this table.

^bTwo-sided χ^2 test.

Table 2

Association between *PUMA* genotypes and SCCHN risk by tumor site

<i>PUMA</i> Variant	Controls ^a n=318 (%)	Overall (n=370)		Oropharynx (n=184)		Non-oropharynx (n=186)	
		Patients (%)	OR ^b (95%CI)	Patients (%)	OR ^b (95%CI)	Patients (%)	OR ^b (95%CI)
rs3810294							
CC	248 (78.0)	262 (70.8)	1.00	128 (69.6)	1.00	134 (72.0)	1.00
CT	64 (20.1)	96 (26.0)	1.4 (1.0–2.1)	49 (26.6)	1.7 (1.0–2.6)	47 (25.3)	1.2 (0.7–1.8)
TT	6 (1.9)	12 (3.2)	2.2 (0.8–6.2)	7 (3.8)	2.7 (0.8–9.6)	5 (2.7)	1.6 (0.5–5.7)
			<i>P</i> _{trend} =0.027		<i>P</i> _{trend} =0.026		<i>P</i> _{trend} =0.134
CT+TT	70 (22.0)	108 (29.2)	1.5 (1.0–2.1)	56 (30.4)	1.7 (1.1–2.7)	52 (28.0)	1.2 (0.8–1.9)
rs2032809							
GG	142 (44.7)	130 (35.1)	1.00	68 (37.0)	1.00	63 (33.9)	1.00
GA	147 (46.2)	178 (48.1)	1.3 (1.0–1.9)	89 (48.4)	1.3 (0.9–2.0)	87 (46.8)	1.4 (0.9–2.1)
AA	29 (9.1)	62 (16.8)	2.2 (1.3–3.7)	27 (14.7)	1.7 (0.9–3.3)	36 (19.4)	2.8 (1.5–5.1)
			<i>P</i> _{trend} =0.001		<i>P</i> _{trend} =0.030		<i>P</i> _{trend} =0.001
GA+AA	176 (55.4)	240 (64.9)	1.5 (1.1–2.0)	116 (63.0)	1.4 (0.9–2.1)	123 (66.1)	1.6 (1.1–2.4)

PUMA, P53 up-regulated modulator of apoptosis gene; SCCHN, Squamous cell carcinoma of the head and neck; CI, Confidence interval; OR, Odds ratio.

^aThe observed genotype frequencies among the controls were in agreement with Hardy-Weinberg equilibrium (*P* = 0.437 for rs3810294, *P* = 0.231 for rs2032809).

^bORs were adjusted for age, sex, smoking, drinking, and HPV16 serology in logistic regression model.

Table 3
Joint effects of HPV16 seropositivity and *PUMA* genotypes on risk of SCCHN by tumor site

HPV16 Serology	PUMA Variant	Control	Overall (n=370)		Oropharynx (n=184)		Non-oropharynx (n=186)	
		n=318 (%)	Patients (%)	OR ^a (95%CI)	Patients (%)	OR ^a (95%CI)	Patients (%)	OR ^a (95%CI)
rs3810294								
-	CC (Ref.)	214 (67.3)	192 (51.9)	1.00	70 (38.0)	1.00	122 (65.6)	1.00
-	CT+TT	64 (20.1)	78 (21.1)	1.3 (0.9-2.0)	30 (16.3)	1.5 (0.9-2.6)	48 (25.8)	1.2 (0.8-1.9)
+	CC	34 (10.7)	70 (18.9)	2.5 (1.6-4.0)	58 (31.5)	5.1 (3.0-8.4)	12 (6.5)	0.7 (0.3-1.4)
+	CT+TT	6 (1.9)	30 (8.1)	6.5 (2.6-16.2)	26 (14.1)	13.8 (5.39-35.5)	4 (2.2)	1.0 (0.3-4.1)
rs2032809								
-	GG (Ref.)	125 (39.3)	97 (26.2)	1.00	38 (20.7)	1.00	60 (32.3)	1.00
-	GA+AA	153 (48.1)	173 (46.8)	1.5 (1.0-2.1)	62 (33.7)	1.4 (0.8-2.2)	110 (59.1)	1.5 (1.0-2.3)
+	GG	17 (5.4)	33 (8.9)	2.8(1.4-5.4)	30 (16.3)	5.2 (2.5-10.6)	3 (1.6)	0.4 (0.1-1.6)
+	GA+AA	23 (7.2)	67 (18.1)	4.3 (2.4-7.4)	54 (29.4)	8.1 (4.3-15.0)	13 (7.0)	1.3 (0.6-2.8)

HPV, Human papillomavirus; *PUMA*, P53 up-regulated modulator of apoptosis gene; SCCHN, Squamous cell carcinoma of the head and neck; CI, Confidence interval; OR, Odds ratio.
^aORs were adjusted for age, sex, and tobacco smoking and alcohol drinking in logistic regression model.

Joint effects of HPV16 seropositivity and *PUMA* genotypes on risk of oropharyngeal cancer stratified by smoking, drinking status, and age

Table 4

HPV16 Serology	Genotypes	Never smokers		Ever smokers		Adjusted OR (95% CI) ^a	
		Patients (n=60)	Controls (n=91)	Patients (n=124)	Controls (n=227)	Never smokers	Ever smokers
<i>PUMA_rs3810294</i>							
-	CC (Ref.)	19	68	51	146	1.00	1.00
-	CT+TT	8	15	22	49	2.3 (0.8-6.5)	1.3 (0.7-2.4)
+	CC	22	7	36	27	12.8 (4.4-37.0)	3.8 (2.1-7.0)
+	CT+TT	11	1	15	5	59.3 (6.8-519.7)	9.2 (3.1-27.1)
<i>PUMA_rs2032809</i>							
-	GG (Ref.)	7	37	31	88	1.00	1.00
-	GA+AA	20	46	42	107	2.2 (0.8-6.0)	1.2 (0.7-2.0)
+	GG	15	3	15	14	30.0 (6.4-141.2)	2.8 (1.2-6.5)
+	GA+AA	18	5	36	18	22.7 (5.8-88.2)	6.2 (3.0-12.8)
		Never drinkers		Ever drinkers		Adjusted OR (95% CI) ^a	
Patients (n=36)	Controls (n=89)	Patients (n=148)	Controls (n=229)	Never drinkers	Ever drinkers		
<i>PUMA_rs3810294</i>							
-	CC (Ref.)	10	61	60	153	1.00	1.00
-	CT+TT	7	19	23	45	2.5 (0.80-7.67)	1.4 (0.8-2.5)
+	CC	12	7	46	27	12.7 (3.8-42.6)	4.1 (2.3-7.3)
+	CT+TT	7	2	19	4	27.2 (4.6-160.7)	11.3 (3.7-34.9)
<i>PUMA_rs2032809</i>							
-	GG (Ref.)	6	35	32	90	1.00	1.00
-	GA+AA	11	45	51	109	1.5 (0.5-4.6)	1.3 (0.8-2.2)
+	GG	5	1	25	16	32.5 (3.2-345.2)	3.9 (1.8-8.4)
+	GA+AA	14	8	40	15	12.9 (3.5-47.9)	7.2 (3.5-14.9)
		Age 58	Age > 58		Adjusted OR (95% CI) ^a		
Patients (n=118)	Controls (n=145)	Patients (n=66)	Controls (n=173)	Age 58	Age > 58		
<i>PUMA_rs3810294</i>							

HPV16 Serology	Genotypes	Never smokers		Ever smokers		Adjusted OR (95% CI) ^a	
		Patients (n=60)		Controls (n=91)		Patients (n=124)	
						Controls (n=227)	Ever smokers
-	CC (Ref.)	39	100	31	114	1.00	1.00
-	CT+TT	16	27	14	37	1.6 (0.7-3.3)	1.7 (0.7-3.1)
+	CC	43	17	15	17	6.7 (3.3-13.5)	3.7 (1.6-8.4)
+	CT+TT	20	1	6	5	58.3 (7.4-458.6)	5.0 (1.4-18.0)
<i>PUMA_rs2032809</i>							
-	GG (Ref.)	20	61	18	64	1.00	1.00
-	GA+AA	35	66	27	87	1.6 (0.8-3.1)	1.1 (0.6-2.2)
+	GG	23	10	7	7	7.3 (2.9-18.6)	3.6 (1.1-12.0)
+	GA+AA	40	8	14	15	15.7 (6.1-40.5)	3.8 (1.5-9.7)

HPV, Human papillomavirus; *PUMA*, P53 up-regulated modulator of apoptosis gene; SCCHN, squamous cell carcinoma of the head and neck; CI, confidence interval; OR, odds ratio.
^aORs were adjusted for age, sex, and tobacco smoking or alcohol drinking where it is proper in logistic regression model.