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Combined effects of the *p53* codon 72 and *p73* G4C14-to-A4T14 polymorphisms on the risk of HPV16-associated oral cancer in never-smokers

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Because p53 and p73 are associated with critical cellular processes and can be inactivated or degraded by the human papillomavirus (HPV) E6 oncoprotein, we investigated the combined effects of *p53* codon 72 and *p73* G4C14-to-A4T14 polymorphisms on the risk of HPV16-associated oral cancer. We analyzed genotype data from 326 patients with squamous cell carcinoma of the oral cavity or oropharynx and 349 cancer-free controls. We found that HPV16 seropositivity was associated with an increased risk of oral cancer [adjusted odds ratio (OR), 3.42; 95% confidence interval (CI), 2.28–5.13], especially among never-smokers (adjusted OR, 8.20; 95% CI, 3.66–18.4) and subjects with variant genotypes [adjusted OR for *p53* Arg/Pro + Pro/Pro (Pro carriers), 5.00; 95% CI, 2.72–9.21; adjusted OR for *p73* GC/AT + AT/AT (AT carriers), 3.83; 95% CI, 1.98–7.41]. HPV16 seropositivity was also associated with an significantly increased risk of oral cancer in all three risk groups with combined genotypes [adjusted ORs (95% CIs) were 2.28 (1.15–4.54) for *p53* Arg/Arg and *p73* GC/GC, the low-risk group; 3.97 (2.14–7.36) for *p53* Arg/Arg and *p73* AT carriers or *p53* Pro carriers and *p73* GC/GC, the medium-risk group and 5.11 (2.00–13.0) for *p53* Pro carriers and *p73* AT carriers, the high-risk group]. Moreover, HPV16-seropositive never-smokers in the high-risk group exhibited an ∼11-fold greater risk of oral cancer (adjusted OR, 11.3; 95% CI, 1.22–106.0) than did HPV16-seronegative never-smokers in the low-risk group. These findings suggest that the combined variants of *p53* and *p73* significantly increase the risk of HPV16-associated oral cancer, especially among never-smokers.

Introduction

The incidence rate of oral cancer has been increasing in recent decades, particularly in young adults (1,2). Epidemiological evidence indicates that high-risk human papillomavirus (HPV) plays an etiological role in and accounts for this continually rising incidence rate, especially in the populations lacking the known risk factors of tobacco and alcohol (3–7). HPV16, the most common of the known HPV types, contributes to carcinogenesis primarily through encoded E6 and E7 oncoproteins (7–9). Although HPV infection plays a major role in the etiology of oral cancer (9,10), only a small fraction of those exposed to high-risk HPV for long periods of time develop oral cancer. This implies that variants in the host genes that are involved in cell cycle control and apoptosis and that interact with HPV oncoproteins E6 or E7 may contribute to interindividual variations in susceptibility to HPV-associated oral cancer. Therefore, the identification of factors that modulate the risk of oral cancer could help identify subgroups of at-risk individuals who would benefit from primary prevention and HPV vaccination programs.

Abbreviations: CI, confidence interval; HPV, human papillomavirus; OR, odds ratio; PCR, polymerase chain reaction.

Cell cycle control is crucial for normal growth and differentiation and maintaining genome stability by monitoring the order and integrity of cell division events. Both *p53* and *p73* are critical cell cycle regulatory tumor suppressor genes that control cell cycle progression via regulation of several important genes and induce apoptosis or G_1 cell cycle arrest (11,12). p53 is the guardian of the genome (13), and the activation of p53, upon cellular stresses such as DNA damage or oncogenic signals from environmental insult, ultimately leads to cell cycle arrest, cellular DNA repair, senescence or apoptosis, thereby protecting normal cells from malignant transformation (14–17). p73, a homology of p53, activates the promoters of several p53-responsive genes participating in DNA repair, cell cycle control and apoptosis, and it inhibits cell growth in a p53-like manner by inducing apoptosis or G_1 cell cycle arrest (18–21). Although direct mutations can alter or inactivate p53 or p73, interactions with the oncogene products of oncogenic viruses (e.g. HPV) can also cause aberrations in p53 and p73 regulatory activity (16,22). The potential of oncogenic HPV for malignant transformation is attributed to its oncoproteins, E6 and E7 (23). The E6 oncoprotein can bind to p53 and promote ubiquitination and rapid proteasome-mediated degradation (24,25). On the other hand, p73 can also be bound to and inactivated by HPV16 E6, but it is resistant to degradation as p53 (22,26). It is possible that *p53* and *p73* variants can alter the affinity for or functional interactions of the E6 protein with both p53 and p73 and thus alter the risk for HPV16-associated carcinogenesis.

Both the *p53* and *p73* genes are highly polymorphic, but the functional effects for many of these polymorphisms are not known. We have previously reported that polymorphisms of *p53* in codon 72 and of *p73* at positions 4 and 14 of exon 2, either alone or in combination, are significantly associated with an increased risk for lung and head and neck cancers (27–30). More recently, we have reported that both these polymorphisms have modifying effects on the risk of HPV16-associated oropharyngeal cancer, particularly in never-smokers (31,32).

To test the hypothesis that individuals who carry a higher number of variants of *p53* codon 72 and *p73* G4C14-to-A4T14 polymorphisms have a higher risk of HPV16-associated oral cancer, we analyzed the combined effect of these two functional polymorphisms of *p53* and *p73* on oral cancer risk in a hospital-based case–control study of 326 non-Hispanic white case subjects with incident oral cancer and 349 cancer-free control subjects who were frequency matched by age, sex, ethnicity, smoking and drinking status. With the information on genotyping data, combination of the variants of the two polymorphisms may provide more comprehensive estimates of the risk of HPV16-associated oral cancer.

Materials and methods

Study subjects

All patients with histologically confirmed squamous cell carcinoma of the oral cavity and oropharynx were consecutively recruited through the Head and Neck Surgery Clinic at The University of Texas M. D. Anderson Cancer Center between April 1996 and June 2002, as part of a molecular epidemiologic study of squamous cell carcinoma of the head and neck. Patients with second primary tumors; primary tumors of the sinonasal tract, nasopharynx, hypopharynx and larynx; primary tumors outside the upper aerodigestive tract; cervical metastases of unknown origin or histopathologic diagnoses other than squamous cell carcinoma were excluded. The accrual rate was 81% for the cases.

Control subjects included one group of 160 (45.8%) cancer-free individuals selected from a control pool of enrollees at the Kelsey-Seybold Clinic,

a multispecialty physician practice with multiple clinics throughout the Houston metropolitan area; the overall response rate for this control group was ~75%. A second control group had 189 (54.2%) cancer-free individuals recruited from visitors who were accompanying cancer patients to the outpatient clinics at M. D. Anderson Cancer Center but who were genetically unrelated to the cases enrolled; the response rate for this control group was ~80%. Control subjects had no previous histories of any cancer, were not receiving therapy or being treated for any diseases and were frequency matched to cases by age (± 5 years), sex, smoking and drinking status. To avoid confounding due to ethnic characteristics, we included only non-Hispanic whites in both case and control groups.

Participants who had smoked >100 cigarettes in their lifetimes were categorized as 'ever-smokers'; the rest were 'never-smokers'. Participants who had drunk alcoholic beverages at least once a week for >1 year were categorized as 'ever-drinkers'; the rest were 'never-drinkers'. After signing informed consent forms, which had been approved by the institutional review boards of both Kelsey-Seybold Clinic and M. D. Anderson Cancer Center, study participants completed a questionnaire regarding demographic and cancer-related risk factors and donated 30 ml of blood. The detailed methods of recruiting participants for this case–control study have been described elsewhere (30,33).

HPV16 serological testing

HPV16 L1 virus-like particles generated from recombinant baculovirus-infected insect cells were used to test for antibodies against HPV16 in the plasma of study subjects by using a standard enzyme-linked immunosorbent assay, as described previously (34,35). Control sera known to be positive and negative were also tested in parallel with the study samples in duplicate on each plate. The cutoff level, above which optical density values were considered positive and below which optical density values were considered negative for HPV16, was based on the absorbance value of a standard pooled serum known to be at the threshold of detection. Samples that were within 15% of the cutoff were tested twice more; those that tested positive in all three runs were considered positive. To eliminate potential binding interference by heparin, we treated the plasma samples with 43 U/ml heparinase I (Sigma, St Louis, MO) before testing (36). We tested serum and heparinized plasma from three randomly selected individuals and did not detect any differences between the reactions of these two samples. We also randomly selected 10% of the samples to retest for confirmation of the original findings, and the results were 100% concordant.

p53 and p73 Genotyping

Blood samples were subjected to centrifugation so that leukocyte cell pellets could be obtained from the buffy coat of 1 ml of each subject's whole blood. Then, genomic DNA was extracted from each pellet using the Qiagen DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

We used polymerase chain reaction (PCR) restriction fragment length polymorphism analysis to identify the *p53* polymorphism at codon 72 with the primers 5′-ATCTACAGTCCCCCTTGCCG-3′ and 5′-GCAACTGACCGTGCAAGTCA-3′. The PCR was performed in 25 µl volumes containing ~50 ng of genomic DNA template, 12.5 pmol of each primer, 0.1 mM of each deoxynucleoside triphosphate, 1 × PCR buffer (50 mM KCl, 10 mM Tris–HCl and 0.1% Triton X-100), 1.5 mM $MgCl_2$ and 1.5 U of Taq polymerase (Promega Corporation, Madison, WI). PCR amplification involved an initial denaturation step at 94°C for 4 min, 35 cycles of 94°C for 40 s, 56°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min. Then, the PCR product (a 296 bp fragment) was digested by *BstU* I (New England BioLabs, Beverly, MA) overnight at 60°C and resolved on 2.5% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME) with ethidium bromide and photographed with Polaroid film. The *p53* 72Pro allele, which lacked the *BstU* I restriction site, had only a single 296 bp band, whereas *p53* 72Arg, which had the *BstU* I restriction site, produced 169 and 127 bp bands.

We tested samples for the *p73* G4C14-to-A4T14 genotypes by PCR with confronting two-pair primers, which makes genotyping possible by electrophoresis without restriction digestion (37). The A4T14 allele was amplified with primers F1 (5′-CCACGGATGGGTCTGATCC-3′) and R1 (5′-GGCCTCCAAGGGCAGCTT-3′), which produced a 270 bp fragment, and the G4C14 allele was amplified with primers F2 (5′-CCTTCCTTCCTGCAGAGCG-3′) and R2 (5′-TTAGCCCAGCGAAGGTGG-3′), which amplified a 193 bp fragment. F1 and R2 also produced a common 428 bp fragment in each PCR. The PCR was performed in 10 µl volumes containing ~20 ng of genomic DNA, 0.1 mM each deoxynucleoside triphosphate, 1 × PCR buffer (50 mM KCl, 10 mM Tris–HCl and 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.5 U of Taq polymerase (Sigma–Aldrich Biotechnology, St Louis, MO) and 2 pmol of each of four primers. The amplification conditions included 10 min of initial denaturation at 95°C, 35 cycles of 1 min at 95°C, 45 s at 62°C and 1 min at 72°C, and a final 5 min extension at 72°C.

All PCR products were visualized on 2% agarose gel containing a 0.25 mg/ml of ethidium bromide. More than 10% of the samples were retested randomly for both polymorphisms, and the results were also 100% concordant.

Statistical analysis

The differences between cases and controls in the distributions of selected demographic variables (age and sex), smoking status, drinking status, *p53* and *p73* allele and genotype frequencies and HPV16 status, were evaluated using the χ^2 test. Both univariate and multivariable logistic regression analyses were used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for cases and controls, stratified by age, sex, smoking status, drinking status and *p53* and *p73* genotypes. In the multivariable logistic regression model, ORs and 95% CIs were adjusted by age, sex, smoking and drinking status. Because only a small number of individuals were homozygous for the Pro or AT allele, *p53* and *p73* genotype data were both dichotomized according to a dominant model, in which homozygosity for Arg/Arg or GC/GC was coded as 0 and both the heterozygosity and homozygosity for Arg/Pro + Pro/Pro or GC/AT + AT/AT were coded as 1. We evaluated the association between the combined genotypes of these two polymorphisms and the risk of HPV16-associated oral cancer with further stratification by smoking status. All tests were two sided. The significance levels for all tests were set at $P < 0.05$. All the statistical analyses were performed using Statistical Analysis System software (Version 9.1; SAS Institute, Cary, NC).

Results

In the final analysis of the two polymorphisms, a total of 326 cases and 349 controls, all with complete *p53* and *p73* genotype data, were included. All cases and controls were self-reported non-Hispanic whites. The distributions of demographic variables and risk factors for the study populations are summarized in Table I. The cases and

Table I. Frequency distribution of demographic and risk factors in oral cancer cases and controls

Variables	Cases (n = 326)		Controls (n = 349)		P-value[a]
	No.	%	No.	%	
Age (years)					
≤40	31	9.5	37	10.6	0.505
41–55	126	38.7	129	37.0	
56–70	119	36.5	141	40.4	
>70	50	15.3	42	12.0	
Sex					
Male	242	74.2	272	77.9	0.259
Female	84	25.8	77	22.1	
Tobacco smoking					
Ever	227	69.6	242	69.3	0.935
Never	99	30.4	107	30.7	
Alcohol drinking					
Ever	250	76.7	256	73.4	0.318
Never	76	23.3	93	26.6	
p53[b]					
Arg/Arg	183	56.1	181	51.9	0.519
Arg/Pro	121	37.1	144	41.2	
Pro/Pro	22	6.8	24	6.9	
Arg/Pro + Pro/Pro	143	43.9	168	48.1	
p73[c]					
GC/GC	195	59.8	214	61.3	0.919
GC/AT	111	34.1	115	33.0	
AT/AT	20	6.1	20	5.7	
GC/AT + AT/AT	131	40.2	135	38.7	
HPV16					
Positive	100	30.7	43	12.3	<0.001
Negative	226	69.3	306	87.7	

[a]Two-sided χ^2 test.
[b]$P = 0.519$ for *p53* genotype distributions; $P = 0.393$ for *p53* allele frequency; the observed genotype frequencies among controls were in agreement with Hardy–Weinberg equilibrium ($p^2 + 2pq + q^2 = 1$) ($P = 0.518$).
[c]$P = 0.919$ for *p73* genotype distributions; $P = 0.709$ for *p73* allele frequency; the observed genotype frequencies among controls were in agreement with Hardy–Weinberg equilibrium ($p^2 + 2pq + q^2 = 1$) ($P = 0.387$).

controls appeared to be adequately frequency matched for age, sex, smoking and drinking status ($P = 0.505$, $P = 0.259$, $P = 0.935$ and $P = 0.318$, respectively). These variables were further adjusted in later multivariable logistic regression analyses to control for any residual effects. The distributions of *p53* and *p73* genotypes among the controls were in agreement with Hardy–Weinberg equilibrium ($P = 0.518$ for *p53* and $P = 0.387$ for *p73*). When comparing the respective genotype distributions between cases and controls, no significant differences were found ($P = 0.519$ for *p53* and $P = 0.919$ for *p73*). However, HPV16 seropositivity was significantly more common in cases than in controls ($P < 0.001$).

In this study, we first examined the association between the two polymorphisms of *p53* and *p73* and the risk of oral cancer, and no any significant associations were found (data not shown). We then utilized information from the same biologic pathway to perform meaningful combination of the two polymorphisms to focus on modifying effect of the combined genotypes on the association between HPV16 seropositivity and risk of oral cancer. The distributions of *p53* and *p73* genotypes, age, sex, smoking and drinking status as stratified by HPV16 status and their associations with oral cancer risk are summarized in Table II. In the overall sample, HPV16 seropositivity was significantly associated with a risk of oral cancer more than three times higher (adjusted OR, 3.42; 95% CI, 2.28–5.13) than HPV16 seronegativity, after adjusting for age, sex, smoking and drinking status. Individuals with the *p53* Arg/Pro + Pro/Pro variant genotypes were associated with a greater risk of HPV16-associated oral cancer (adjusted OR, 5.00; 95% CI, 2.72–9.21) than were individuals with the *p53* Arg/Arg wild-type genotype (adjusted OR, 2.65; 95% CI, 1.52–4.63). Similarly, the *p73* GC/AT + AT/AT variant genotypes were also associated with a higher risk of HPV16-associated oral cancer (adjusted OR, 3.83; 95% CI, 1.98–7.41) than was the *p73* GC/GC wild-type genotype (adjusted OR, 3.20; 95% CI, 1.91–5.36).

To investigate the effect of other factors on the risk of HPV16-associated oral cancer, we stratified the associations between HPV16 status and oral cancer risk by age, sex, smoking and drinking status. We found that the risk of oral cancer associated with HPV16 seropositivity was elevated in all subgroups, particularly among individuals <56 years old (adjusted OR, 4.93; 95% CI, 2.77–8.77), men (adjusted OR, 3.96; 95% CI, 2.50–6.29), never-smokers (adjusted OR, 8.20; 95% CI, 3.66–18.4) and never-drinkers (adjusted OR, 6.22; 95% CI, 2.45–15.8). However, the interaction between HPV16 status (seropositive versus seronegative) and sex, drinking status and combined genotypes was not statistically significant ($P = 0.117$ for sex; $P = 0.334$ for drinking status and $P = 0.129$ for combined genotypes) except for age ($P = 0.018$).

Because there was no significant interaction effect between these two polymorphisms (*p53* variant genotypes versus *p73* variant genotypes) ($P_{int.} = 0.635$) on the risk of HPV16-associated oral cancer, we categorized subjects into three combined genotype groups based on the level of HPV16-associated oral cancer risk linked to the genotypes. This allowed us to evaluate the combined effects of the *p53* and *p73* polymorphisms on the risk of HPV16-associated oral cancer. Subjects with *p53* Arg/Arg and *p73* GC/GC genotypes were placed in the low-risk group, subjects with *p53* Arg/Arg and *p73* AT carriers or *p53* Pro carriers and *p73* GC/GC genotypes were placed in the medium-risk group and subjects with *p53* Pro carriers and *p73* AT carriers were placed in the high-risk group. As shown in Table III, after adjusting for age, sex, smoking and drinking status, HPV16 seropositivity was associated with a significantly increased risk of HPV16-associated oral cancer in the low-risk group (adjusted OR, 2.28; 95%

Table II. Association of selected variables with risk of HPV16-associated oral cancer

Variables	HPV16 status	Cases ($n = 326$)		Controls ($n = 349$)		Adjusted OR (95% CI)[a]
		No.	%	No.	%	
All subjects	−	226	69.3	306	87.7	1.00
	+	100	30.7	43	12.3	3.42 (2.28–5.13)
p53 genotypes						
Arg/Arg	−	135	73.8	156	86.2	1.00
	+	48	26.2	25	13.8	2.65 (1.52–4.63)
Arg/Pro + Pro/Pro	−	91	63.6	150	89.3	1.00
	+	52	36.4	18	10.7	5.00 (2.72–9.21)
p73 genotypes						
GC/GC	−	136	69.7	187	87.4	1.00
	+	59	30.3	27	12.6	3.20 (1.91–5.36)
GC/AT + AT/AT	−	90	68.7	119	88.1	1.00
	+	41	31.3	16	11.9	3.83 (1.98–7.41)
Age (years)						
≤55	−	98	60.5	152	88.4	1.00
	+	64	39.5	20	11.6	4.93 (2.77–8.77)
>55	−	128	78.0	154	87.0	1.00
	+	36	22.0	23	13.0	2.16 (1.18–3.93)
Sex						
Male	−	159	65.7	239	87.9	1.00
	+	83	34.3	33	12.1	3.96 (2.50–6.29)
Female	−	67	79.8	67	87.0	1.00
	+	17	20.2	10	13.0	1.60 (0.67–3.84)
Smoking						
Never	−	60	60.6	97	90.7	1.00
	+	39	39.4	10	9.3	8.20 (3.66–18.4)
Ever	−	166	73.1	209	86.4	1.00
	+	61	26.9	33	13.6	2.48 (1.53–4.01)
Drinking						
Never	−	51	67.1	84	90.3	1.00
	+	25	32.9	9	9.7	6.22 (2.45–15.8)
Ever	−	175	70.0	222	86.7	1.00
	+	75	30.0	34	13.3	2.89 (1.83–4.59)

[a]HPV positive versus HPV negative in each stratum; ORs were adjusted for age, sex, smoking and drinking status in logistic regression models.

Table III. Association of combined *p53* and *p73* genotypes with risk of HPV16-associated oral cancer

Risk group[a]	HPV16 status	Cases (n = 326)		Controls (n = 349)		Adjusted OR (95% CI)[b]
		No.	%	No.	%	
Low-risk group	−	81	72.3	92	84.4	1.00
	+	31	27.7	17	15.6	2.28 (1.15–4.54)
Medium-risk group	−	109	70.8	159	89.8	1.00
	+	45	29.2	18	10.2	3.97 (2.14–7.36)
High-risk group	−	36	60.0	55	87.3	1.00
	+	24	40.0	8	12.7	5.11 (2.00–13.0)

[a]Low-risk group: individuals with *p53* Arg/Arg and *p73* GC/GC genotypes; medium-risk group: individuals with *p53* Arg/Arg and *p73* AT carriers or *p53* Pro carriers and *p73* GC/GC and high-risk group: individuals with *p53* Pro carriers and *p73* AT carriers.
[b]HPV positive versus HPV negative in each stratum; ORs were adjusted for age, sex, smoking and drinking status in logistic regression models.

CI, 1.15–4.54) and the medium-risk group (adjusted OR, 3.97; 95% CI, 2.14–7.36). However, among individuals in the high-risk group, HPV16 seropositivity was associated with an even higher risk of oral cancer (adjusted OR, 5.11; 95% CI, 2.00–13.0).

Because we found a significant interaction between HPV16 status (seropositive versus seronegative) and smoking status (ever-smoking versus never-smoking) on the risk of oral cancer (P = 0.026), we further evaluated the association between the combined genotypes of these two polymorphisms and the risk of HPV16-associated oral cancer as stratified by HPV16 serological status and smoking status. In the stratified analysis, we found that the elevated risk of HPV16-associated oral cancer in all three combined genotype groups was higher among never-smokers than among ever-smokers (Table IV). HPV16-seropositive never-smokers in the high-risk group exhibited an ∼11-fold greater risk of oral cancer (adjusted OR, 11.3; 95% CI, 1.22–106.0) than did HPV16-seronegative never-smokers in the low-risk group. Also, HPV16 seropositivity in never-smokers in the low-risk group (adjusted OR, 3.55; 95% CI, 1.04–12.1) and medium-risk group (adjusted OR, 6.99; 95% CI, 2.00–24.4) conferred an ∼3.5 and 7-fold greater risk of oral cancer, respectively. However, HPV16-seropositive ever-smokers in the high-risk group exhibited only an ∼3-fold greater risk of oral cancer (adjusted OR, 2.88; 95% CI, 1.10–7.56) than did HPV16-seronegative ever-smokers in the low-risk group.

Discussion

In this hospital-based case–control study of a non-Hispanic white population, we confirmed that HPV16 seropositivity was significantly associated with an increased risk of oral cancer. Both *p53* Arg/Pro + Pro/Pro and *p73* GC/AT + AT/AT variant genotypes were associated with a greater risk of HPV16-associated oral cancer than were the *p53* Arg/Arg and *p73* GC/GC wild-type genotypes, respectively. The HPV16-associated risk of oral cancer was higher among never-smokers than among ever-smokers for all three combined risk groups, and the joint risk was particularly high among individuals in the high-risk group who were HPV16 seropositive and never-smokers. These findings suggest that the *p53* codon 72 and *p73* G4C14-to-A4T14 polymorphisms may have joint effects on the risk of HPV16-associated oral cancer, particularly in never-smokers.

Although the precise mechanism by which the polymorphisms play a role in the development of HPV16-associated oral cancer has not yet been clarified, there are some biologically plausible explanations. For example, p53 and p73 play similar roles in the regulation of cell cycle control, DNA repair and apoptosis (14–21). Both can interact with HPV16 by being directly bound to and inactivated by oncoprotein E6 (22,24,25,38). Furthermore, there are biological interactions between members of the p53 family (39), which may interact with each other, as previous studies have indicated that p53 and p73 do (40–42). In some human malignancies associated with *p53* mutation, higher levels of p73 expression were found in cancer tissues than in adjacent normal tissues, which implies that p73 may compensate for the loss of p53 function (40,41). In *p73* and *p63* knockout mice, p53 is completely inactive (42). Additionally, the p53 and p73 proteins have similar domain structures, very high amino acid identities in the DNA-binding domain and some common target genes, including p21 and Bax (39). Therefore, p53 and p73 may have substantially similar biological effects, which may co-operatively contribute to the risk of HPV16-associated oral cancer.

The *p53* codon 72 polymorphism results in a substitution of proline (Pro) for arginine (Arg) in the p53 protein sequence (43), which may alter the apoptotic potential of p53 and the susceptibility of p53 to E6-mediated degradation (44–47), thereby affecting the carcinogenic potential of HPV16. The *p73* G4C14-to-A4T14 polymorphism lies upstream of the initiating AUG of exon 2 and may form a stem-loop structure, which may result in an alteration of gene expression, possibly by altering the efficiency of translational initiation (48). The altered *p73* gene expression may lead to an altered interaction between the E6 protein and p73, thus modulating the risk of HPV-associated cancer (49). Currently, no reported studies have investigated the association between this *p73* polymorphism and the risk of HPV16-associated oral cancer, but several studies have found that the *p53* codon 72 polymorphism alters the susceptibility of p53 to oncogenic HPV E6-mediated degradation and is associated with an increased risk of HPV-associated cancers (31,47,50,51). These studies suggest that p73 may have a similar effect to that of p53 on the development of HPV16-associated oral cancer. Together, these findings of similar biological effects, the interaction between p53 and p73 proteins and the interaction of p53 and p73 with HPV oncogenic proteins suggest that *p73* and *p53* polymorphisms have a joint effect on susceptibility to HPV16-associated oral cancer.

The findings of the present study are consistent with those of our previous study (31) and an Italian study (50), both of which showed that the *p53* Pro/Pro genotype is associated with a higher risk of HPV16-associated squamous cell carcinoma of the oropharynx. Other studies have also found that the *p53* Pro/Pro genotype is associated with an increased risk of cervical cancer, which is mainly caused by HPV, compared with the Arg/Arg genotype (52,53). However, some other studies did not find significant associations between this *p53* polymorphism and the risk of cervical cancer (47,51). These inconsistent results may be caused by several factors, including the involvement of different HPV subtypes, inclusion of different ethnic groups and use of different study designs. Thus far, only a few epidemiological studies have investigated the association of *p73* G4C14-to-A4T14 polymorphism with the risk of HPV16-associated cancers. Our current case–control analysis found this *p73* polymorphism to be significantly associated with an increased risk of squamous cell carcinoma of the oropharynx, particularly in never-smokers (32). A Japanese study reported an association of this *p73* polymorphism with an increased risk of cervical cancer, although that finding was not significant (54). However, because no studies that we are aware of have investigated the combined effects of these two functional polymorphisms on the risk of HPV16-associated oral cancer, we evaluated the combined effects of these two polymorphisms on the risk of

Table IV. Stratification of risk of oral cancer in never-smokers and ever-smokers by combined *p53* and *p73* genotypes and HPV16 exposure

Smoking status and risk group[a]	HPV16 status	Cases (n = 326)		Controls (n = 349)		Crude OR (95% CI)	Adjusted OR (95% CI)[b]
		No.	%	No.	%		
Never-smokers		(n = 99)		(n = 107)			
Low-risk group	−	22	22.2	26	24.3	1.00	1.00
	+	13	13.1	5	4.7	3.07 (0.95–9.97)	3.55 (1.04–12.1)
Medium-risk group	−	29	29.3	52	48.6	0.66 (0.32–1.36)	0.59 (0.28–1.27)
	+	19	19.2	4	3.7	5.61 (1.66–19.0)	6.99 (2.00–24.4)
High-risk group	−	9	9.1	19	17.8	0.56 (0.21–1.49)	0.50 (0.18–1.37)
	+	7	7.1	1	0.9	8.27 (0.94–72.5)	11.3 (1.22–106.0)
Ever-smokers		(n = 227)		(n = 242)			
Low-risk group	−	59	26.0	66	27.3	1.00	1.00
	+	18	7.9	12	4.9	1.68 (0.75–3.77)	1.85 (0.81–4.23)
Medium-risk group	−	80	35.2	107	44.2	0.84 (0.53–1.32)	0.76 (0.48–1.22)
	+	26	11.5	14	5.8	2.08 (0.99–4.35)	1.89 (0.87–4.04)
High-risk group	−	27	11.9	36	14.9	0.84 (0.46–1.54)	0.78 (0.42–1.46)
	+	17	7.5	7	2.9	2.72 (1.05–7.00)	2.88 (1.10–7.56)

[a]Low-risk group: individuals with *p53* Arg/Arg and *p73* GC/GC genotypes; medium-risk group: individuals with *p53* Arg/Arg and *p73* AT carriers or *p53* Pro carriers and *p73* GC/GC and high-risk group: individuals with *p53* Pro carriers and *p73* AT carriers.
[b]ORs were adjusted for age, sex, smoking and drinking status in logistic regression models.

HPV16-associated oral cancer as a reasonable next step. As we found in our previous study in lung cancer (27), individuals who possess a greater number of *p53* and *p73* risk alleles may be at higher risk for developing HPV16-associated oral cancer.

In this study, we also found that the combined effect of these two polymorphisms in never-smokers was associated with a much higher risk of HPV16-associated oral cancer than in ever-smokers. For instance, HPV16-seropositive never-smokers in the high-risk group exhibited an ∼11-fold greater risk of oral cancer than did HPV16-seronegative never-smokers in the low-risk group, whereas HPV16-seropositive ever-smokers in the high-risk group had only a <3-fold increased risk of oral cancer compared with HPV16-seronegative ever-smokers in the low-risk group. This finding implies that the combined genotypes of these two polymorphisms may jointly have a stronger interaction with HPV16 among never-smokers than among ever-smokers. Therefore, it may be important to stratify study groups by smoking status when evaluating the effects of HPV on risk of oral cancer in future epidemiological studies. In addition, we found that the risk of HPV16-associated oral cancer was higher in younger subjects (<56 years old), men, never-smokers and never-drinkers. These results are in line with the notion that HPV-associated cancers are more common in young adults, never-smokers and never-drinkers and that oral cancers occur much more frequently in men than in women (6,55–57). These findings also indicate that HPV16 and tobacco and alcohol use may not be cofactors in the development of oral cancer.

Our findings imply that the evaluation of multiple polymorphisms of different genes in the same pathways may improve the precision of cancer risk estimates. However, interpretation of these findings may be limited for several reasons. First, we investigated only one functional single-nucleotide polymorphism per gene in this current study, but both *p53* and *p73* have several other functional single-nucleotide polymorphisms in the likely functional regions of the two genes. Secondly, stratified analyses included a limited number of individuals in some subgroups. Therefore, our results could be a chance finding and should be confirmed in larger studies. Moreover, selection bias could not be ruled out as a factor because this was a hospital-based case–control study, and controls were not selected from the same population as cases. As our study included only non-Hispanic whites, it is uncertain whether our results are generalizable to other ethnic populations. However, because the cases and controls were frequency matched by age, sex, ethnicity, smoking and drinking status and no statistically significant differences in the frequency of *p53* and *p73* genotypes were found between cases and controls, we believe that the effects of any confounding factors might have been minimized. Finally, although we acknowledge that HPV16 seropositivity might not reflect actual tumor HPV16 status, an early study has confirmed a reasonable concordance between HPV16 seropositivity and HPV16 DNA positivity of tumor tissues (9), and more importantly, the use of serologic status allows for the inclusion of a cancer-free control group.

In conclusion, this study provides evidence that the simultaneous presence of the *p53* codon 72 polymorphism and *p73* G4C14-to-A4T14 polymorphism may have joint effects on the risk of HPV16-associated oral cancer in non-Hispanic whites, particularly in never-smokers. However, because this is the first study concerning the combined effects of the *p53* and *p73* polymorphisms on the risk of HPV16-associated oral cancer, further studies with larger sample sizes are needed to confirm our findings.

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