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Genetic variations in *TERT–CLPTM1L* genes and risk of squamous cell carcinoma of the head and neck

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Single-nucleotide polymorphisms (SNPs) of *TERT*-rs2736098 (C > T) and *CLPTM1L*-rs401681(C > T) at the 5p15.33 locus are significantly associated with cancer risk as reported in genome-wide association studies (GWAS), but there are no reported studies for squamous cell carcinoma of the head and neck (SCCHN). In a case–control study of 1079 SCCHN cases and 1115 cancer-free controls of non-Hispanic whites who were frequency matched by age and sex, we genotyped for these two SNPs and assessed their associations with SCCHN risk. Compared with the CC genotypes of each polymorphism, the associations of a slightly reduced risk of SCCHN with the variant genotypes of CT + TT of both polymorphisms were approaching statistical significance [Odds ratio (OR) = 0.90, 95% confidence interval (CI) = 0.76–1.08 for *TERT*-rs2736098 and OR = 0.86, 95% CI = 0.71–1.04 for *CLPTM1L*-rs401681, respectively]. When the two SNPs were combined, the variant genotypes of the two SNPs were significantly associated a moderately reduced risk of SCCHN (OR = 0.82, 95% CI = 0.67–0.99), and the number of variant genotypes was associated with a significantly reduced risk in a dose–response manner ($P = 0.028$). Furthermore, the reduced risk was more pronounced in ever smokers, ever drinkers and patients with oropharyngeal cancer. Our results suggested that these two SNPs at the 5p15.33 locus may be associated with a reduced risk of SCCHN, particularly for their combined effect. Although we added additional evidence for the association of the two SNPs with cancer risk as reported in GWAS, additional studies are needed to replicate our findings.

Introduction

The chromosome 5p15.33 locus contains two well-known genes, telomerase reverse transcriptase (*TERT*) and cleft lip and palate transmembrane 1-like (*CLPTM1L*) (also called *CRR9*), which have been implicated in carcinogenesis. The *TERT* gene is the reverse transcriptase component of telomerase and is essential for the maintenance of telomere DNA length, chromosomal stability and cellular immortality (1–4). *TERT* is in a large condensed chromosomal region that begins at the 3′-end of the *CLMPT1L* gene and extended beyond the *XTRP2* gene (2,5–7). Normally, *TERT* messenger RNA (mRNA) is only expressed in embryonic stem cells and germ cells, which is undetectable or can be detected at very low levels in most somatic cells; however, abnormal expression of *TERT* mRNA and protein has been observed in many tumors, including squamous cell carcinoma of the head and neck (SCCHN), suggesting a critical role of *TERT* in tumorigenesis (8–14). Although the function of the *CLPTM1L* gene is largely unknown, studies have demonstrated that it may induce apoptosis. For example, CLPTM1L, as a predicted transmembrane protein, is upregulated in cisplatin-resistant ovary cancer cell line and might be involved in the apoptotic response of cells under genotoxic stress of cisplatin. Therefore, the overexpression of CLPTM1L might induce apoptosis in cisplatin-sensitive cells and lung cells under genotoxic stress caused by tobacco-related carcinogens (15). Recently, several genome-wide association studies (GWAS) have reported that common polymorphisms of *TERT* and *CLPTM1L* are associated with risk of many types of cancer (16–19). For example, a synonymous coding single-nucleotide polymorphism (SNP, A305A, rs2736098) in exon 2 of *TERT* and another SNP (rs401681) in intron 13 of *CLPTM1L* located at the 5p15.33 locus (18) were reportedly to be associated with risk of basal cell carcinoma, lung, bladder and prostate cancers, although the known environmental factors may also play a role in the risk, such as tobacco smoking for lung and bladder cancer and ultraviolet irradiation for basal cell carcinoma.

SCCHN, which includes cancers of the oral cavity, pharynx and larynx, is one of the sixth most common cancers worldwide (20). In the USA, ∼48 000 new cases were diagnosed and resulted in 11 000 deaths in 2009 (20,21). Although smoking and alcohol drinking are well-established risk factors for SCCHN and human papillomavirus (HPV) infection has been suggested to be a main risk factor specifically for oropharyngeal carcinoma (22,23), genetic variations, such as SNPs, may also modify the risk of SCCHN.

To date, studies have demonstrated that *CLPTM1L* mRNA is overexpressed in laryngeal squamous cell carcinoma (24) and that the *TERT* gene plays an important role in the etiology of human oral cancer (14), but no study has evaluated the association between *TERT*-rs2736098 and *CLPTM1L*-rs401681 and risk of SCCHN. Therefore, given an important role for these two SNPs in the development of many cancers as identified in GWAS studies, we hypothesized that these two genetic variants may contribute to SCCHN risk. To test this hypothesis, we genotyped these two SNPs and analyzed their associations with risk of SCCHN in a case–control study of 1079 cases and 1115 cancer-free controls in a US non-Hispanic white population.

Abbreviations: CI, confidence interval; CLPTM1L, cleft lip and palate transmembrane 1-like; GWAS, genome-wide association studies; HPV, human papillomavirus; LD, linkage disequilibrium; mRNA, messenger RNA; OR, odds ratio; SCCHN, squamous cell carcinoma of the head and neck; SNP, single-nucleotide polymorphism; TERT, telomerase reverse transcriptase.

Material and methods

Study subject

The details of cases and controls have been described previously (25). Briefly, the study population included 1079 non-Hispanic white subjects with newly diagnosed untreated primary tumors of the oral cavity (n = 316; 29.3%), oropharynx (n = 548; 50.8%) or larynx (n=172; 15.9%) and hypopharynx (n = 43; 4%) in the period of October 1999 and October 2007; by using frequency matching on age, sex and ethnicity, 1115 cancer-free control subjects were identified from the visitors who accompanied patients to The University of Texas M. D. Anderson Cancer Center clinics in the same time period. Patients with second SCCHN primary tumors, primary tumors of the nasopharynx or sinonasal tract or any histopathological diagnosis other than SCCHN were excluded. After having provided a written informed consent, each eligible subject was interviewed for additional information about risk factors, such as tobacco smoking and alcohol use and a one-time sample of 30 ml of blood for biomarker tests. The research protocol was reviewed and approved by our institutional review board.

Genotyping analysis

From each blood sample, a leukocyte cell pellet obtained from the buffy coat was obtained for DNA extraction by using the QIAGEN DNA Blood Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. As shown in Figure 1 for the location of the *TERT*-rs2736098 in exon 2 and *CLPTM1L*-rs401681in intron 13 sites at chromosome 5p15.33 (http://hapmap.ncbi.nlm.nih.gov), the linkage disequilibrium (LD) plot shows regions of chromosome 5p15.33 around 20 kb upstream of *TERT* and downstream of

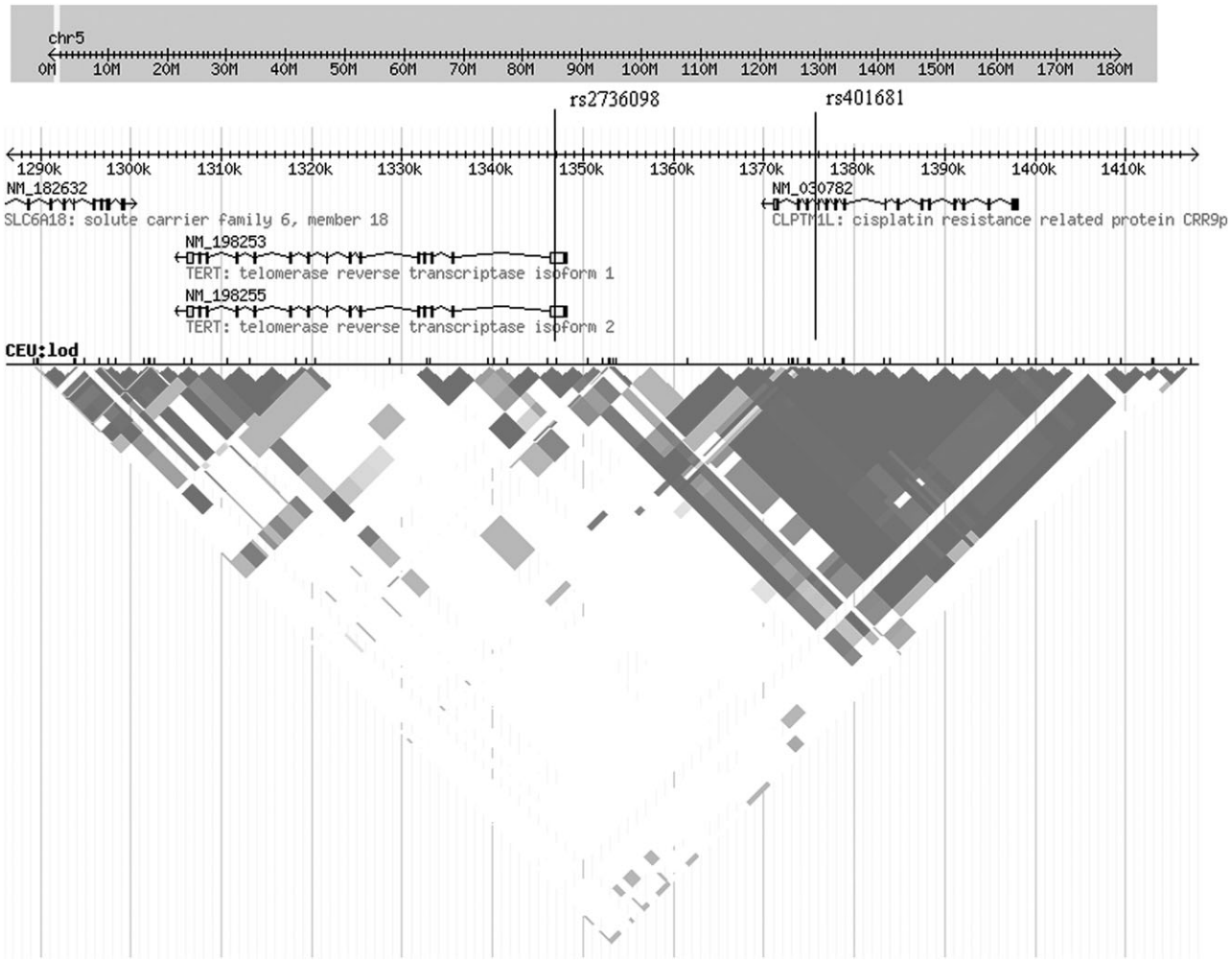

Fig. 1. The location of the *TERT*-rs2736098 (C > T) and *CLPTM1L*-rs401681(C > T) sites at chromosome 5p15.33 obtained from http://hapmap.ncbi.nlm.nih.gov. The LD plot shows region of chromosome 5p15.33 around 20 kb upstream of *TERT* and downstream of *CLMPT1L* between 5p15.33:1,286,287 and 5p15.33:1,418,002. The rs2736098 is located in exon 2 of *TERT* and rs401681 is located in intron 13 of *CLPTM1L.*

CLMPT1L between 5p15.33:1,286,287 and 5p15.33: 1,418,002. These two independent SNPs (*TERT*-rs2736098 and *CLPTM1L*-rs401681) were genotyped using the TaqMan methodology in 384-well plates and read with the Sequence Detection Software on an ABI-Prism7900 instrument according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Primers and probes were supplied by Applied Biosystems. Each plate included four negative controls (no DNA), duplicated commercial positive controls (TaqMan Control Genomic DNA; Applied Biosystems) and eight repeats samples. Amplification was done under the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. For all genotypes, the assay success rate was >99% and the repeated sample's results were 100% concordant.

Statistical analysis

The differences in selected demographic variables, smoking and alcohol consumption between SCCHN cases and controls were evaluated by using the χ^2-test. The associations of genotypes of *TERT* and *CLPTM1L* with risk of SCCHN were estimated by computing the odds ratios (ORs) and their 95% confidence intervals (CIs) from both univariate and multivariable logistic regression models in case–control analysis. These analyses were performed with or without adjustment for age (in years), sex, smoking status and alcohol use. Those subjects who had smoked at least 100 cigarettes in their lifetime were defined as ever smokers; otherwise, they were considered never-smokers. Subjects who drank alcoholic beverages at least once a week for >1 year were defined as 'ever drinkers' and the rest as 'never-drinkers. All tests were two sided, and $P < 0.05$ was considered significant. Considering potential joint effect of the two SNPs, we evaluated the main effect of the combined genotypes of both polymorphisms on risk of SCCHN. Stratified analysis for individual or in combination of the two SNPs was performed to estimate risk for subgroups by age, sex, smoking status, drinking status and tumor site. All statistical analyses were performed with the SAS software (version 9.1.3; SAS Institute, Cary, NC).

Results

The distribution of selected characteristics of the cases and controls is presented in Table I. All subjects were non-Hispanic whites, and there were no significant differences in the distributions of age and sex between the cases and the controls ($P = 0.623$ and 0.495, respectively) with a similar mean age (57.1 ± 11.2 years for cases and 56.7 ± 11.0 years for controls) ($P = 0.657$). There were more ever smokers and ever drinkers in the cases (72.2 and 72.5%, respectively) than in the controls (51.1 and 56.5%, respectively; $P < 0.001$ for both). However, all these variables were further adjusted for any residual confounding effect in later multivariable logistic regression analyses.

The genotype and allele frequencies of *TERT*-rs2736098 and *CLPTM1L*-rs401681 polymorphisms and their associations with risk of SCCHN are summarized in Table II. The genotype distribution of the two SNPs in the controls was in agreement with that of the Hardy–Weinberg equilibrium ($P = 0.271$ for *TERT* and $P = 0.775$ for *CLPTM1L*). Overall, although we did not observe a significant association of each genotype of the two SNPs with risk of SCCHN, the associations of the variant CT + TT genotypes of each polymorphism with a reduced risk of SCCHN were approaching statistical significance (OR = 0.90; 95% CI = 0.76–1.08 for TERT and OR = 0.86;

Table I. Frequency distributions of selected variables in SCCHN patients and cancer-free controls

Variable	No (%)		P^a
	Cases (n = 1079)	Controls (n = 1115)	
Mean age (±SD)	57.1 (±11.2)	56.7 (±11.0)	0.657
Age (years)			
<57	531 (49.2)	537 (48.2)	0.623
≥57	548 (50.8)	578 (51.8)	
Gender			
Male	812 (75.2)	853 (76.5)	0.495
Female	267 (24.8)	262 (23.5)	
Smoking status			
Ever	779 (72.2)	570 (51.1)	<0.001
Never	300 (27.8)	545 (48.9)	
Alcohol use			
Ever	782 (72.5)	630 (56.5)	<0.001
Never	297 (27.5)	485 (43.5)	
Tumor site			
Oropharynx	548 (50.8)		
Non-oropharynx[b]	531 (49.2)		

[a]Two-sided χ^2 test for differences in the distributions between the cases and controls.
[b]Included oral cavity (316), larynx (n = 172) and hypopharynx (n = 43).

Table II. Genotype frequencies of the *TERT* and *CLPTM1L* polymorphisms among SCCHN cases and control subjects and their associations with risk of SCCHN

Genotypes	No (%)		P	Adjusted OR (95% CI)[b]
	Cases (n = 1079)	Controls (n = 1115)[a]		
TERT-rs2736098 (C > T)				
CC	588 (54.5)	576 (51.7)	0.411[c]	1.00
CT	419 (38.8)	461 (41.3)		0.91 (0.76–1.10)
TT	72 (6.7)	78 (7.0)		0.85 (0.60–1.22)
P Trend				0.237
CT + TT	491 (45.5)	539 (48.3)	0.184[d]	0.90 (0.76–1.08)
T allele frequency	0.261	0.277	0.238[e]	
CLPTM1L-rs401681(C > T)				
CC	357 (33.1)	333 (29.9)	0.142[c]	1.00
CT	495 (45.9)	557 (49.9)		0.82 (0.67–1.00)
TT	227 (21.0)	225 (20.2)		0.98 (0.76–1.25)
P Trend				0.605
CT + TT	722 (66.9)	782 (70.1)	0.104[d]	0.86 (0.71–1.04)
T allele frequency	0.440	0.452	0.431[e]	
Trichotomize combined[f]				
0	123 (11.4)	105 (9.4)		1.00
1	699 (64.8)	699 (62.7)	0.327[g]	0.86 (0.64–1.16)
2	257 (23.6)	311 (27.9)	0.046[g]	0.72 (0.52–0.99)
P Trend				0.028
Dichotomize combined[f]				
0–1	822 (76.2)	804 (72.1)		1.00
2	257 (23.8)	311 (27.9)	0.048[g]	0.82 (0.67–0.99)

[a]The observed genotype frequency among the control subjects was in agreement with the Hardy–Weinberg equilibrium ($p^2 + 2pq + q^2 = 1$) (χ^2 = 1.212, P = 0.271 for *TERT* C > T; χ^2 = 0.082, P = 0.775 for *CLPTM1L* C > T).
[b]Adjusted for age, sex, smoking and drinking status.
[c]Two-sided χ^2 test for difference in frequency distribution of genotype between cases and controls.
[d]Two-sided χ^2 test for distribution of combined genotypes.
[e]Two-sided χ^2 test for allele distribution.
[f]0 group, *TERT* CC genotype and *CLPTM1L* CC genotype; 1 group, *TERT* CC genotype and *CLPTM1L* variant genotypes or *CLPTM1L* CC genotype and *TERT* variant genotypes; 2 group, *TERT* variant genotypes and *CLPTM1L* variant genotypes.
[g]P values were obtained in a logistic regression model adjusted for age, sex, smoking and drinking status.

95% CI = 0.71–1.04 for *CLPTM1L*) compared with the corresponding CC genotype. Because each of these two SNPs appeared to have a minor effect on risk of SCCHN, we then combined the two SNPs to evaluate their joint effect on risk of SCCHN. As shown in Table II, we categorized all genotypes of each SNP into the three or two different groups (trichotomized and dichotomized) according to the number of variant genotypes. For trichotomized groups, individuals with 1 or 2 variant genotypes had a significantly decreased risk for SCCHN, compared with individuals with 0 variant genotype, and the trend in risk associated with the number of protective genotypes was statistically significant (P_{trend} = 0.028). The individuals with 2 variant genotypes had a significantly decreased risk for SCCHN (adjusted OR = 0.82, 95% CI = 0.67–0.99), compared with individuals with 0 or 1 variant genotypes in dichotomized groups.

We also investigated the individual and combined effects of the two polymorphisms by subgroups of age, sex, smoking and alcohol status and tumor sites with adjustment for aforementioned variables shown in Table III. We found that, compared with the CC wild-type homogenous genotype, individuals with variant genotypes of *CLPTM1L*-rs401681 exhibited a close to significant or significant reduction in risk of SCCHN in ever smokers (P = 0.070), ever drinkers (P = 0.082) and patients with oropharyngeal cancer (P = 0.003), but these association was not observed for *TERT*-rs2736098. However, individuals carrying 2 variant genotypes of the two SNPs had a significantly reduced risk of SCCHN, compared with those with 0 or 1 variant genotype, in ever smokers (P = 0.008), ever drinkers (P = 0.010) and patients with oropharyngeal cancer (P = 0.050).

Discussion

To our knowledge, this is the first study that has investigated whether the two GWAS-identified genetic variants (*TERT*-rs2736098 and *CLPTM1L*-rs401681) at the 5p15.33 locus are associated with SCCHN risk in a non-Hispanic white population. Overall, although only near borderline significant associations were observed between each of the two SNPs and SCCHN risk, we found that the combined variant genotypes of both SNPs were significantly associated with a reduced risk of SCCHN in a protective genotype dose–response manner. This risk was more evident in subgroups of ever smokers, ever drinkers and patients with oropharyngeal cancer. These finding suggested that the two SNPs of the 5p15.33 locus may have a joint effect on the risk of SCCHN, particularly oropharyngeal cancer. Although the exact mechanism by which these variants influence the risk of SCCHN is not clear, it is possible that these variants may be either functional itself or it may be in LD with other functional variants that are involved in the etiology of SCCHN.

The *TERT* gene has been mapped to chromosome 5p15.33 and consisted of 16 exons and 15 introns spanning 35 kb of genomic DNA, (5) and the TERT protein is a catalytic subunit and a key regulator of telomerase activity. Overexpression of TERT and telomerase activity has been observed in many tumors, which possibly contribute to unlimited cell division and carcinogenesis. It has also been reported that telomerase may play a role in tumor progression and metastasis by activation of the glycolytic pathway and in suppression of tumor cell differentiation (26,27). Although the TERT expression has been well documented to be involved in the tumorigenesis of various cancers, including SCCHN (8–14), relatively few association studies have been conducted to evaluate the role of the *TERT* polymorphism in cancer development. To date, only three studies have investigated the association between the *TERT*-rs2736098 polymorphism and cancer risk. For example, in a Polish study of 1995 breast cancer patients and 2296 controls, the *TERT* T allele was associated with a borderline significant association with risk of breast cancer, but

Table III. Stratification analysis of associations of the genotypes and combined of the *TERT* and *CLPTM1L* with risk of SCCHN

Stratified	*No.*	Adjusted OR (95% CI)[a]								
	(Case/controls)	*TERT* rs2736098: C > T			*CLPTM1L* rs401681: C > T			Combined variant genotypes		
		CC	CT + TT	P^b	CC	CT + TT	P^b	0–1	2	P^b
Age										
≤57	531/537	1.00	0.95 (0.74–1.22)	0.676	1.00	0.76 (0.58–1.01)	0.057	1.00	0.80 (0.61–1.07)	0.133
≥57	548/578	1.00	0.87 (0.68–1.12)	0.280	1.00	0.95 (0.73–1.23)	0.702	1.00	0.83 (0.63–1.11)	0.211
Gender										
Male	812/853	1.00	0.94 (0.77–1.15)	0.533	1.00	0.86 (0.69–1.06)	0.164	1.00	0.87 (0.69–1.10)	0.235
Female	267/262	1.00	0.81 (0.56–1.17)	0.258	1.00	0.87 (0.58–1.30)	0.482	1.00	0.68 (0.45–1.02)	0.062
Smoking status										
Ever	779/570	1.00	0.88 (0.70–1.10)	0.254	1.00	0.80 (0.63–1.02)	0.070	1.00	0.70 (0.54–0.91)	0.008
Never	300/545	1.00	0.96 (0.72–1.28)	0.788	1.00	0.91 (0.67–1.25)	0.573	1.00	0.99 (0.72–1.36)	0.957
Alcohol use										
Ever	782/630	1.00	0.87 (0.69–1.10)	0.206	1.00	0.81 (0.64–1.03)	0.082	1.00	0.72 (0.55–0.92)	0.010
Never	297/485	1.00	0.99 (0.74–1.33)	0.958	1.00	0.92 (0.67–1.26)	0.601	1.00	1.01 (0.73–1.40)	0.961
Tumor site										
Oropharynx	548/1115	1.00	0.97 (0.79–1.20)	0.798	1.00	0.71 (0.57–0.89)	0.003	1.00	0.79 (0.62–1.01)	0.055
Non-oropharynx[c]	531/1115	1.00	0.82 (0.65–1.03)	0.086	1.00	1.11 (0.87–1.43)	0.409	1.00	0.84 (0.65–1.10)	0.181

[a]Adjusted by age, sex, smoking and drinking status.
[b]Two-sided χ^2 test for difference in frequency distribution of genotype between cases and controls by adjusted for age, sex, smoking and alcohol consumption.
[c]Included oral cavity (316), larynx (n = 172) and hypopharynx (n = 43).

in stratified analysis, the author found that the T allele was associated with reduced risk of breast cancer among individuals with a family history of breast cancer (28). In a GWAS of 11 290 cancer patients and 31 162 controls, it was shown that the *TERT* T variant genotypes were associated with significantly increased risk of basal cell carcinoma, lung cancer, bladder cancer and prostate cancer in Caucasian populations (18). More recently, a Korea population study of 720 lung cancer patients and 720 cancer free controls found that the *TERT* T variant genotypes were associated with significantly increased risk of lung cancer (29). In the present case–control study, we only found a near borderline significant association between *TERT* T variant genotypes and SCCHN risk. Little is known about the effect of *TERT* T allele on TERT expression or telomerase activity. Although *TERT* T allele was associated with shorter telomeres with nominal significance (P = 0.027), no association was observed between *TERT* T allele and the RNA expression of *TERT* (18). Therefore, further investigations of the potential effects of *TERT* T allele on the functions and expression of *TERT* are warranted. These conflicting results from above epidemiological association studies lead us to think that while the *TERT*-rs2736098 T variant allele may be functionally relevant, the epidemiologic findings may also imply its LD with other functional variants of *TERT* or with alleles at other nearby loci of other genes. However, this hypothesis remains to be tested. Furthermore, the effect of a low-penetrance susceptibility gene on disease risk might be influenced by other modifying genes and environmental factors through gene–gene or gene–environment interactions. Therefore, different genetic backgrounds and different risk factors might explain, to some extent, the somewhat conflicting results in risk estimates associated with this *TERT* polymorphism in different cancers and different populations. Other factors in the studies such as small sample size, inclusion of a single polymorphism or inadequate adjustment for confounding factors could also cause the inconsistent results.

The *CLPTM1L* gene may play a role in apoptotic response, and overexpression of *CLPTM1L* mRNA has been observed in many cancer types, including SCCHN (18,24,30,31). Although several studies of associations between the *CLPTM1L*-rs401681polymorphism and cancers have been published (16–19), some inconsistent results exist. For example, an early study reported that the *CLPTM1L* T genotypes were not associated with risk of lung cancer in 341 cases and 431 controls in a Caucasian populations (15), but another association study of 2396 lung cancer cases and 3001 controls found that the *CLPTM1L* T allele was associated with a significantly decreased risk of lung cancer (17). However, in the recent GWAS of 20 726 cancer patients and 134 650 controls, Rafnar *et al.* (18) reported that the *CLPTM1L* C genotypes were associated with significantly increased risk of lung cancer, basal cell carcinoma, bladder cancer and prostate cancer. More recently, in another GWAS of 3468 basal cell carcinoma patients and over 38 107controls, the *CLPTM1L* C allele was associated with significantly increased risk of basal cell carcinoma, but a protective effect on melanoma risk was reported in a study of 3843 cutaneous melanoma patients and 41 963 controls (19). In the present study, although a borderline significant association was observed between the *CLPTM1L* polymorphism and risk of SCCHN, we found a significantly reduced risk of oropharyngeal cancer, but not for non-oropharyngeal cancer including oral cavity, hypopharyngeal or laryngeal cancers. These differences are likely to be driven by risk factors associated with tumor subsites, with non-oropharyngeal cancer mainly associated with smoking and alcohol but oropharyngeal cancer largely associated with HPV. Therefore, our data suggest that *CLPTM1L* variant genotypes may play different roles in the etiology of two different tumor subsites. However, the exact functional relevance of *CLPTM1L*-rs401681 SNP located in intron 13 of *CLPTM1L* remains unclear, although it may be in strong LD with other potential functional or causal SNPs contributing to the risk of SCCHN. These hypotheses need to be further tested. Finally, the mechanisms underlying the combined variant genotypes of both *TERT* and *CLPMT1L* polymorphisms were significantly associated with risk of SCCHN, particularly for oropharyngeal cancer, warrant additional investigations in the future.

There are several limitations in this study. The possible selection bias could not be ruled out because this was a hospital-based case–control study and the controls were not selected from the same population from which the cases may occur. Also, because our analysis was limited to non-Hispanic white subjects, it is uncertain whether these results are generalizable to other populations. However, by matching on age, sex and ethnicity, potential confounding factors might be minimized. Due to the retrospective nature of the original study design, we did not have reliable information on HPV infection and the observed significant association between genetic polymorphisms and risk of oropharyngeal subsite could be also due to chance as the sample size for this subsite was relatively small.

In summary, these two polymorphisms may contribute individually or jointly to reduce risk of SCCHN, particularly oropharyngeal cancer, in ever smokers and ever drinkers. Additional larger and

prospective population-based studies with detailed information about HPV infection are needed to further confirm our findings.

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