CASP3 Polymorphisms and Risk of Squamous Cell Carcinoma of the Head and Neck

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Abstract

Purpose: Caspase-3 plays a central role in executing cell apoptosis and thus in carcinogenesis, but little is known about the role of *CASP3* variants in susceptibility to SCCHN.

Experimental Design: Genotype and haplotypes of the first intron (rs4647601:G>T and rs4647602:C>A) and 5′-untranslated region (UTR; rs4647603:G>A) of *CASP3* (NT_022792.17) were determined for 930 SCCHN patients and 993 cancer-free controls in a U.S. non-Hispanic white population. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated in multivariate logistic regression analysis.

Results: We found that the *CASP3* rs4647601:TT variant genotype was associated with an increased risk of SCCHN (adjusted OR, 1.32; 95% CI, 1.00-1.73) compared with the GG genotype. This risk was more evident in the subgroups of younger (≤56 years) subjects, males, and never smokers with a significant trend for increased risk with increased number of variant T allele ($P < 0.05$ for all). However, these risks were not found for other two SNPs. Furthermore, individuals with two copies of haplotypes TCG or GCA were found to have a significant increased risk of SCCHN (OR, 1.31; 95% CI, 1.07-1.61) compared with the other haplotypes, and this risk was more evident in less advanced diseases (OR, 1.45; 95% CI, 1.11-1.89) than in the advanced diseases (OR, 1.22; 95% CI, 0.96-1.54).

Conclusions: These results suggested that genetic variation in *CASP3* may contribute to SCCHN risk. Larger studies are needed to confirm our findings.

Head and neck cancer is one of the most common cancers in the world (1). It is estimated that there were approximately 40,566 new cases of squamous cell carcinoma of the head and neck (SCCHN) in the United States in 2007 (2). Although tobacco and alcohol consumptions are primary risk factors for SCCHN (3, 4), recent molecular epidemiologic studies suggest that genetic variations or single nucleotide polymorphisms (SNP) may also contribute to individual susceptibility to cancer (5, 6). Among these genetic variations, SNPs in apoptosis-regulatory genes have been shown to affect the propensity for carcinogenesis in many cancer types (7). However, the role of SNPs in major apoptosis-regulatory caspase genes in SCCHN remains understudied.

Apoptosis is a programmed cell death process under both normal physiologic and pathologic conditions. Caspases, which are a family of cysteine-dependent aspartate-specific proteases, are important mediators for the apoptotic process. Caspases cleave numerous intracellular substrates in the initiation of cell dissolution (8). According to their specific functions, caspases can be divided into initiator caspases (e.g., caspase-2, caspase-8, caspase-9, and caspase-10) and effector caspases (e.g., caspase-3, caspase-6, and caspase-7), and they play important roles in the extrinsic and intrinsic apoptosis pathways, respectively (9–13). External cell death signals are received by cell surface receptors, such as death receptors 4 and 5, leading to activation of initiator caspase-8 and caspase-10 (14). Similarly, intrinsic or mitochondrial death-signaling pathway initiated by the release of cytochrome *c* also results in activation of the other initiator caspase-9 (15, 16). Activated initiator caspases subsequently activate the downstream effector caspase-3, caspase-6, and caspase-7 (15–19).

Caspase-3 activation plays a central role in the execution phase of cell apoptosis. Somatic mutations in *CASP3* have been reported in human cancer tissues and cell lines (20, 21), including gastric cancer, non–small cell lung carcinoma, and hepatocellular carcinoma (22–24). Several published studies showed associations between caspase SNPs and cancer risk (25–28), with only one study investigating the association between *CASP3* and non–Hodgkin's lymphoma (25). To date, mutations of *CASP3* in SCCHN have not been reported.

We hypothesized that SNPs in *CASP3* may contribute to susceptibility to SCCHN and disease progression. To test this hypothesis, we conducted a case-control study of 930 patients with SCCHN and 993 cancer-free controls in a U.S. non-Hispanic white population. We genotyped three SNPs located in the first intron (rs4647601:G>T and rs4647602:C>A) and

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Translational Relevance

The establishment of genetic variation in *CASP3* as a risk factor for squamous cell carcinoma of the head and neck (SCCHN) risk is an etiologically important step in predicting risk in the general population for further identification of individuals at risk for primary prevention. Indeed, this study found one of the three *CASP3* single nucleotide polymorphisms (SNP) to be associated with risk of SCCHN, particularly in younger subjects, male, and never smokers with less advanced SCCHN, suggesting that this SNP was a marker for susceptibility to but not disease progression of SCCHN.

5′-UTR (rs4647603:G>A) regions because variations in this region most likely affect their gene expression.

Materials and Methods

Study subjects. The detailed methods of recruiting cases and controls have been described elsewhere (29). This case-control analysis included 930 patients with histologically confirmed SCCHN between October 1999 and February 2006, including cancers of the oral cavity, oropharynx, hypopharynx, and larynx, identified at The University of Texas M. D. Anderson Cancer Center. Patients with the second SCCHN primaries, primaries of the nasopharynx or sinonasal tract, primaries outside the upper aerodigestive tract, cervical metastases of unknown origin, or histopathologic diagnoses other than squamous cell carcinoma were excluded. All cases were non-Hispanic whites and had not received any radiotherapy or chemotherapy at the time of recruitment and blood donation. The response rate of eligible cases was 90%.

According to the American Joint Committee on Cancer (30), the regional lymph node involvement of SCCHN was defined as N_0 to N_3 as follows: N_0, no regional node metastasis; N_1, metastasis in a single ipsilateral lymph node, ≤3 cm in the greatest dimension; N_2, metastasis in a single ipsilateral lymph node, >3 cm but <6 cm in the greatest dimension, or in multiple ipsilateral lymph nodes, none ≥6 cm in the greatest dimension, or in any bilateral or contralateral lymph node, <6 cm in the greatest dimension; N_3, metastasis in any lymph node, ≥6 cm in the greatest dimension. The extent of the primary SCCHN was defined as T_1 to T_4 as follows: T_1, tumor ≤2 cm at the greatest dimension; T_2, tumor >2 cm but <4 cm in the greatest dimension; T_3, tumor ≥4 cm in the greatest dimension; T_4, tumor invading adjacent structures.

The 993 cancer-free subjects we recruited in the same time period were genetically unrelated visitors or companions of patients seen at M. D. Anderson clinics, who were frequency matched to the cases by age (±5 y), sex, and ethnicity. The response rate of eligible controls whom we approached for recruitment was 85%. After being asked to sign an informed consent form, all subjects enrolled in the study were interviewed to gather demographic data and history of smoking and alcohol use. Each eligible subject donated 30 mL of blood collected in heparinized tubes to be used for biomarker assays, including DNA extraction and genotyping. The research protocol was approved by the M. D. Anderson Institutional Review board.

SNP selection. The National Center for Biotechnology Information SNP database was used to identify potentially functional SNPs of *CASP3*,[6] in which there were at least 181 reported SNPs (NT_022792.17); however, there was no common (minor allele frequency ≥0.05) nonsynonymous SNPs in the coding region or common SNPs in the known promoter region. Therefore, we decided to select common SNPs in the 5′ transcriptional regulatory region before the translation starting codon, which may alter the transcription of *CASP3*. Although exons 1 and 2 of the *CASP3* gene do not code for amino acids, these first two exons and the first intron likely contribute to transcription activity of *CASP3*. Based on these considerations, three common SNPs located in exon 2 (5′-UTR; rs4647603:G>A) and intron 1 (rs4647601:G>T and rs4647602:C>A) of *CASP3* were selected for genotyping.

Genotyping. From each blood sample, a leukocyte cell pellet obtained from the buffy coat by centrifugation of 1 mL of whole blood was used for DNA extraction. Genomic DNA was isolated with the Qiagen DNA Blood Mini kit (Qiagen, Inc.) according to the manufacturer's instructions. RFLP-PCR was used to identify *CASP3* (rs4647601:G>T, rs4647602:C>A, and rs4647603:G>A) polymorphisms. Each PCR was done in a 25-mL reaction mixture containing ~50 ng of genomic DNA template, 12.5 pmol of each primer, 0.1 mmol/L of each deoxynucleotide triphosphate, 1× PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, 0.1% Triton X-100), 1.5 mmol/L $MgCl_2$, and 1.5 units of Taq polymerase (Promega Corp.). The PCR profile consisted of an initial melting step of 96°C for 5 min; 35 cycles of 96°C for 45 s, 56°C for 40 s, and 72°C for 30 s; and a final extension step of 72°C for 10 min.

The following primers were used: 5′-GCGGTAGCGCCGTCCGTTGC-3′ (forward) and 5′-ACCGAGCTCCGAGGGCGGGAG-3′ (reverse) for *CASP3* (rs4647601:G>T) and 5′-TGTGTATCCGTGGCCACAGCT-3′ (forward) and 5′-GAGAATGGGGGAAGAGGCAGGT-3′ (reverse) for *CASP3* (rs4647603:G>A). The amplified PCR products were 103 and 132 bp for rs4647601:G>T and rs4647603:G>A SNPs, respectively. The *Hpy*ch4V and *Pvu*II restriction enzymes (New England Biolabs) were used to delineate rs4647601:G>T and rs4647603:G>A SNPs, which resulted in 84- and 19-bp fragments or 113- and 19-bp fragments, respectively. The rs4647602:C>A genotypes were determined by the SNPlex method (primer and probes were available on request; Applied Biosystems).

PCR was conducted and the genotype results were evaluated without knowledge of the subjects' case-control status. More than 10% of the samples were randomly selected for repeated assays, and the results were 100% concordant.

Statistical analysis. We used χ^2 test to compare the frequency distributions of the case and control groups by demographic variables, smoking status, alcohol use, each allele, and genotype of the *CASP3* polymorphisms. We also tested the Hardy-Weinberg equilibrium of genotype distributions separately for both patients and control subjects. Additionally, we used unconditional univariate and multivariate logistic regression analysis to examine the associations between variant genotypes and SCCHN risk by estimating odds ratio (OR) and 95% confidence intervals (95% CI) with and without adjustment for age, ethnicity, smoking status, and alcohol use and assessed any trend in the associations.

Linkage disequilibrium was tested between the alleles of the *CASP3* gene and reported using Lewontin's D' and correlation coefficient r^2 among the three *CASP3* SNPs (rs4647601:G>T, rs4647602:C>A, and rs4647603:G>A). Reconstruction of *CASP3* haplotypes using the observed and unphased genotypes of these three SNPs were accomplished by the PHASE software program PHASE 2.0 (31), which estimated the probability of a specific haplotype pair for each individual. We choose the haplotype pair with the highest probability as each individual's haplotype pair or their "diplotype." The haplotype frequency was then used for comparison between the case and control groups using χ^2 test. Multiple logistic regressions were used to analyze haplotype or diplotype associations with risk of SCCHN. To test for the significance of haplotype variations, the likelihood ratio test was used to compare the intercept-only model with the haplotype-plus model. Similarly, we use likelihood ratio test to assess the significant of haplotype effect for risk of SCCHN by adding haplotype into the logistic regression model with age, sex, and use of alcohol and tobacco

[6] http://www.NCBI.NLM.NIH.gov/SNP

Table 1. Frequency distributions of selected variables in SCCHN cases and cancer-free controls

Variables	*n* (%)		*P**
	Cases (*n* = 930)	Controls (*n* = 993)	
Age (y)			
≤56	458 (49.2)	513 (51.7)	0.290
>56	472 (50.8)	480 (48.3)	
Sex			
Female	206 (22.1)	227 (22.9)	0.710
Male	724 (77.9)	766 (77.1)	
Smoking status			
Never	254 (27.3)	483 (48.6)	<0.001
Former	342 (36.8)	362 (36.5)	
Current	334 (35.9)	148 (14.9)	
Alcohol use			
Never	256 (27.5)	448 (45.1)	<0.001
Former	206 (22.2)	157 (15.8)	
Current	468 (50.3)	388 (39.1)	
Tumor site			
Oral cavity	282 (30.3)		
Oropharynx †	498 (53.6)		
Larynx	150 (16.1)		

*Two-sided χ^2 test.
† Including 42 of hypopharynx.

already in the model. ORs were reported for both haplotypes and diplotypes with and without the adjustment of age, sex, tobacco use, and drinking status.

We further stratified the genotype, haplotype, and diplotype data by subgroups of age, sex, smoking, and alcohol drinking and assessed the risk of SCCHN with multivariate logistic regression models. Homogeneity of ORs in the stratified analysis and interactions of paired variables of interest were further tested using the likelihood ratio statistic. All statistical tests were two sided, and a P value of <0.05 was considered significant by using the Statistical Analysis System software (version 8.2; SAS Institute).

Results

The characteristics of the study population are shown in Table 1. Because we used frequency matching on age and sex, there were no significant differences in mean age or sex distribution between 930 cases and 993 controls (P = 0.290 for age and P = 0.710 for sex). However, the cases had more current smokers and drinkers than the controls (35.9% versus 14.9% for current smokers and 50.3% versus 39.1% for current drinkers; P < 0.001 for both smoking and alcohol use). Among the cases, 282 (30.3%) had cancers of the oral cavity, 4,398 (53.6%) of pharynx (including 42 of oropharynx), and 150 (16.1%) of larynx (Table 1).

The genotype and allele distributions of the three selected SNPs in the cases and controls are summarized in Table 2. The observed genotype frequencies for two SNPs were in Hardy-Weinberg equilibrium in the controls (P = 0.377 for rs4647601 and P = 0.917 for rs4647603) but not for the third SNP (P = 0.029 for rs4647602). There was no statistically significant difference in the distributions of either allele or genotype frequencies of these three SNPs (Table 2). However, when compared with the GG genotypes of the *CASP3* rs4647601:G>T, the *CASP3* rs4647601:TT genotype was associated with an increased risk of SCCHN (adjusted OR, 1.32; 95% CI, 1.00-1.73), and there was no association with any genotype of the *CASP3* rs4647602:C>A and rs4647603:G>A SNPs (Table 2).

To identify any susceptible subgroup, we focused on stratified analysis of associations between the *CASP3* rs4647601:G>T and risk of SCCHN by selected variables listed in Table 1 because we did not identify any association with other two SNPs. As shown in Table 3, the significantly increased risk associated with the

Table 2. Logistic regression analysis of associations between *CASP3* polymorphisms and risk of SCCHN

CASP3 variants	*n* (%)		*P*	OR (95% CI)	Adjusted OR (95% CI)*
	Cases (*n* = 930)	Controls (*n* = 993)			
CASP3 (rs4647601:G>T)					
GG	314 (33.8)	365 (36.8)	0.184 †	1.00	1.00
GT	435 (46.8)	463 (46.6)		1.09 (0.89-1.33)	1.08 (0.88-1.33)
TT	181 (19.4)	165 (16.6)		1.28 (0.98-1.65)	1.32 (1.00-1.73)
GT+TT	616 (66.2)	628 (63.2)	0.170 ‡	1.14 (0.95-1.38)	1.14 (0.94-1.39)
T allele frequency	0.428	0.399	0.073§		
CASP3 (rs4647602:C>A)					
CC	802 (86.2)	833 (83.9)	0.180 †	1.00	1.00
AC	122 (13.1)	147 (14.8)		0.86 (0.67-1.12)	0.90 (0.68-1.18)
AA	6 (0.7)	13 (1.3)		0.48 (0.18-1.27)	0.46 (0.17-1.26)
AC+AA	128 (13.8)	160 (16.1)	0.149 ‡	0.83 (0.65-1.07)	0.86 (0.66-1.12)
A allele frequency	0.072	0.087	0.098§		
CASP3 (rs4647603:G>A)					
GG	687 (73.9)	753 (75.8)	0.547 †	1.00	1.00
GA	223 (24.0)	223 (22.5)		1.10 (0.89-1.36)	1.05 (0.84-1.31)
AA	20 (2.1)	17 (1.7)		1.29 (0.67-2.48)	1.34 (0.68-2.68)
GA+AA	243 (26.1)	240 (24.2)	0.322 ‡	1.11 (0.90-1.36)	1.07 (0.86-1.33)
A allele frequency	0.141	0.129	0.298§		

*Adjusted for age, sex, smoking status, and alcohol use in a logistic regression model.
†Two-sided χ^2 test for difference in frequency distribution of genotypes between cases and controls.
‡Two-sided χ^2 test for difference in frequency distribution of combined genotypes between cases and controls.
§Two-sided χ^2 test for difference in frequency distribution of alleles between cases and controls.

Table 3. ORs of SCCHN associated with *CASP3* genotypes in the stratified analysis

Stratified variables	*CASP3* (rs4647601:G>T)					*P* and trend test*
	n (case/control) †	*n* (case/control)	Adjusted OR (95% CI) ‡	*n* (case/control)	Adjusted OR (95% CI) ‡	
	GG	GT	GT vs GG	TT	TT vs GG	
Age (y)						
≤56	150/203	215/228	1.25 (0.93-1.67)	93/82	**1.62 (1.02-2.34)**	0.052/**0.012**
>56	164/162	220/235	0.93 (0.69-1.56)	88/83	1.05 (0.71-1.56)	0.746/0.909
Gender						
Male	245/281	331/364	1.04 (0.82-1.31)	148/121	**1.45 (1.07-1.97)**	0.062/**0.032**
Female	69/84	104/99	1.25 (0.80-1.98)	33/44	0.94 (0.52-1.70)	0.341/0.938
Smoking status						
Never	83/192	116/213	1.27 (0.90-1.80)	55/78	**1.61 (1.04-2.48)**	0.077/**0.028**
Former	104/128	169/177	1.20 (0.86-1.68)	69/57	1.54 (0.99-2.38)	0.197/0.056
Current	127/45	150/73	0.76 (0.48-1.19)	57/30	0.86 (0.48-1.54)	0.261/0.461
Alcohol use						
Never	76/165	118/205	1.21 (0.85-1.74)	62/78	1.59 (0.95-1.85)	**0.044/0.038**
Former	70/56	99/77	0.95 (0.59-1.54)	37/24	1.13 (0.60-2.14)	0.791/0.794
Current	168/144	218/181	1.07 (0.78-1.46)	82/63	1.32 (0.86-2.00)	0.864/0.228
Tumor site						
Oral cavity	92/365	141/463	1.12 (0.82-1.53)	49/165	1.15 (0.76-1.73)	0.438/0.465
Pharynx	171/365	228/463	1.04 (0.81-1.33)	99/165	1.28 (0.93-1.76)	0.275/0.161
Larynx	51/365	66/463	0.96 (0.64-1.47)	49/165	1.36 (0.81-2.30)	0.266/0.330

NOTE: The result is bolded if the 95% CI does not include 1 or $P < 0.05$.
*P value was from the χ^2 test statistics from comparisons of genotype frequencies between cases and controls and P value for the allele trend test obtained from logistic regression analyses.
† n (cases/controls) for each stratum.
‡ ORs were adjusted for all covariates (age, gender, smoking status, and alcohol use), excluding the stratified variable.

CASP3 rs4647601:TT genotype was evident in the subgroups of younger (≤56 years) subjects (adjusted OR, 1.62; 95% CI, 1.02-2.34), males (adjusted OR, 1.45; 95% CI, 1.07-1.97), and never smokers (adjusted OR, 1.61; 95% CI, 1.04-2.48) as well as a borderline increased risk in never drinkers (adjusted OR, 1.59; 95% CI, 0.95-1.85) compared with the common homozygous GG genotype (Table 3). Furthermore, the trend for increased risk with increased number of variant T allele in these subgroups was also statistically significant (Table 3). However, except for age and *CASP3* rs4647601:G>T (P for interaction = 0.049), there was

Table 4. Frequencies of inferred haplotypes and diplotypes of *CASP3* based on the observed genotypes in SCCHN cases and cancer-free control

	Cases, *n* (%)	Controls, *n* (%)	Crude OR	Adjusted OR*
Haplotypes	***n* = 1,860**	***n* = 1,986**		
GCG	705 (37.9)	814 (41.0)	1.00	1.00
TCG	759 (40.8)	745 (37.5)	1.18 (1.02-1.36)	1.18 (1.02-1.37)
GCA	229 (12.3)	215 (10.9)	1.25 (1.01-1.56)	1.21 (0.96-1.51)
MAF < 0.1	167 (9.0)	211 (10.6)	0.89 (0.70-1.11)	0.92 (0.72-1.17)
P value		0.024 †	0.017 ‡	0.050§
Diplotype‖	***n* = 930**	***n* = 993**		
0	227 (24.4)	277 (27.9)	1.00	1.00
1	398 (42.8)	448 (45.1)	1.08 (0.87-1.35)	1.06 (0.84-1.34)
2	305 (32.8)	268 (27.0)	1.39 (1.09-1.77)	1.36 (1.06-1.75)
0 or 1	625 (67.2)	725 (73.0)	1.00	1.00
2	305 (32.8)	268 (27.0)	1.32 (1.09-1.61)	1.31 (1.07-1.61)

Abbreviation: MAF, minor allele frequency.
*Adjusted for sex, age, smoking status, and alcohol use in a logistic regression model.
†Pearson's χ^2 test was used to test for the difference in the distribution of all haplotypes/diplotypes between cases and controls.
‡Likelihood ratio test for the significance of haplotypes associated with SCCHN risk.
§Likelihood ratio test for haplotype effect with adjustment for sex, age, smoking status, and alcohol use.
‖Diplotype was coded as 0 if there was no TCG or GCA in the haplotype pair, 1 if there was only one copy of TCG or GCA in the haplotype pair, and 2 if there were two copies of TCG or GCA in the haplotype pair.

no evidence for possible interaction between other pairs of covariates (data not shown).

Further linkage disequilibrium analysis revealed relatively low incomplete linkage disequilibrium among the three loci in *CASP3* (r^2 = 0.047, D' = 0.878 for rs4647601 and rs4647602; r^2 = 0.046, D' = 0.645 for rs4647601 and rs4647603; and r^2 = 0.01, D' = 0.842 for rs4647602 and rs4647603), suggesting that they may be independent tagging SNPs (i.e., $r^2 < 0.8$ for all pairwise linkage disequilibrium). However, only rs4647603 (but not rs4647601 and rs4647602) is one of the seven tagging SNPs (using $r^2 \geq 0.08$ and minor allele frequency $\geq$0.05 for pairwise linkage disequilibrium) reported in the HapMap, but it only tags rs4647609:T>C located in intron 2 with a minor allele frequency of 0.175 for the C allele.

By using the PHASE 2.0 program, we inferred eight possible haplotypes based on the observed genotype data, of which three common (>10%) haplotypes (GCG, TCG, and GCA) represented 91% of all haplotypes for the cases and 89.4% for the controls (Table 4). The haplotype distribution between the cases and the controls was statistically different (P = 0.024). When the most common haplotype GCG was used as the reference, both TCG and GCA haplotypes were associated with a significant increased risk of SCCHN (OR, 1.18; 95% CI, 1.02-1.36 and OR, 1.25; 95% CI, 1.01-1.56, respectively). In the logistic regression analysis, haplotypes were significantly associated with risk of SCCHN in both a univariate model (P = 0.017) and a multivariate model with adjustment for age, sex, smoking, and alcohol use (P = 0.050; Table 4).

We further inferred diplotypes derived from TCG and GCA as *CASP3* variant haplotypes. A diplotype was coded as "0" if there was no TCG or GCA in the haplotype pair, "1" if there was only one copy of TCG or GCA in the haplotype pair, and "2" if there were two copies of TCG or GCA in the haplotype pair. Two copies of TCG or GCA were associated with a 1.36-fold elevated risk of SCCHN (95% CI, 1.06-1.75) when compared with the diplotype 0 and a 1.31-fold elevated risk (95% CI, 1.07-1.61) when compared with the diplotype "0+1" (Table 4).

To explore whether genetic variation in *CASP3* may be associated with disease progression, we did additional stratified analysis of association between *CASP3* diplotypes (using the dichotomized variable: two copies versus zero + one copies) and risk of SCCHN by primary tumor stage, lymph node metastasis status, and their combination. The results showed that there were significantly increased risk of SCCHN associated with the diplotype of two copies of TCG or GCA haplotypes among patients with T_1 and T_2 (adjusted OR, 1.40; 95% CI, 1.01-1.92 and adjusted OR, 1.30; 95% CI, 0.98-1.71, respectively) or N_0 and N_2 (adjusted OR, 1.35; 95% CI, 1.02-1.78 and adjusted OR, 1.33; 95% CI, 1.03-1.71, respectively) status. When we combined T and N status, this risk was more evident in less advanced diseases (OR, 1.45; 95% CI, 1.11-1.89 for $T_{1,2}$ and N_0) than in the advanced diseases (OR, 1.22; 95% CI, 0.96-1.54 for $T_{3,4}$ and N_{1-3}; Table 5). However, there was no evidence for any interaction between these covariates (data not shown).

Discussion

In this case-control study of SCCHN, we investigated the associations of three SNPs located in the intron 1 (rs4647601:G>T and rs4647602:C>A) and 5′-UTR (rs4647603:G>A) of the apoptosis gene *CASP3* with risk of SCCHN in a U.S. non-Hispanic white population. Among these three SNPs, we found that the *CASP3* rs4647601:TT variant genotype was associated with an increased risk of SCCHN, and this risk was more evident in the subgroups of younger (≤56 years) subjects, males, and never smokers with a significant trend for increased risk with increased number of the variant rs4647601:T allele, but these risks were not observed for other two SNPs. However, diplotypes containing two copies of haplotypes TCG or GCA of these three variants were also associated with increased risk of SCCHN, further suggesting that genetic variation in *CASP3* may contribute to the susceptibility to SCCHN. Because the functionality of the

Table 5. Associations between diplotypes of *CASP3* promoter polymorphisms and progression of SCCHN

Stratified variables*	*CASP3* diplotype		Crude OR	Adjusted OR †	*P* ‡
	n (case/control)	Two copies of TCG or GCA			
Primary tumor (T)					
T_1	221/993	34.4/27.0	**1.42 (1.04-1.94)**	**1.40 (1.01-1.92)**	**0.027**
T_2	327/993	33.0/27.0	**1.33 (1.02-1.75)**	1.30 (0.98-1.71)	**0.036**
T_3	196/993	30.1/27.0	1.17 (0.83-1.63)	1.14 (0.81-1.61)	0.372
T_4	186/993	33.3/27.0	1.35 (0.97-1.89)	1.28 (0.89-1.83)	0.078
Lymph node metastasis					
N_0	343/993	33.5/27.0	**1.37 (1.05-1.78)**	**1.35 (1.02-1.78)**	**0.021**
N_1	135/993	30.4/27.0	1.18 (0.80-1.75)	1.10 (0.74-1.65)	0.409
N_2	415/993	33.0/27.0	**1.33 (1.04-1.71)**	**1.33 (1.03-1.72)**	**0.023**
N_3	37/993	32.4/27.0	1.30 (0.64-2.62)	1.25 (0.61-2.56)	0.465
Combine T and N					
$T_{1,2}$ and N_0	352/993	34.9/27.0	**1.45 (1.12-1.89)**	**1.45 (1.11-1.89)**	**0.005**
$T_{3,4}$ or N_{1-3}	578/993	31.5/27.0	1.24 (0.99-1.56)	1.22 (0.96-1.54)	0.057

NOTE: The result is bolded if the 95% CI does not include 1 or $P < 0.05$.
*Stratified variables: T, the extent of the primary SCCHN (T_1: tumor ≤2 cm at the greatest dimension; T_2-T_4: increasing greatest dimensions); N, regional lymph node involvement (N_0: no regional lymph nodes involved; N_1-N_3: increasing involvement of regional lymph nodes).
†ORs were adjusted for age, gender, smoking status, and alcohol use.
‡*P* value from the χ^2 test of different frequencies of *CASP3* genotypes in cases and controls.

SNPs under study is unknown, some subgroups had fewer observations, and we only used the statistical method to infer the haplotype/diplotype instead of technically proving the cosegregate of the SNPs, our findings need further validation in mechanistic investigation and larger association studies.

Most of the caspase mutations detected in human cancer resulted in reduced apoptotic activities compared with wild-type caspases (32, 33), suggesting that attenuated cellular apoptosis by caspase mutations plays an important role in tumorigenesis. Caspase-3 is known to play a crucial role during apoptosis as an execution-phase caspase, and it is not surprising that the mutation of *CASP3* has been found in human tumor tissues and cell lines (20, 21). Although few studies have investigated the role of genetic alterations of *CASP3* in modulating apoptosis activities, some association studies have suggested possible links between polymorphisms of caspase genes and susceptibility to cancer (25–28), but little is known about the role in SCCHN susceptibility of variants of *CASP3*. To the best of our knowledge, the present study is the first large molecular epidemiologic study on the association between *CASP3* polymorphisms and risk of SCCHN. The only other case-control study investigated the role of two other *CASP3* SNPs in the 3′-UTR region (rs6948:Ex8-280C>A and rs1049216:Ex8-567T>C) in non–Hodgkin's lymphoma susceptibility, and their results showed that these two variants in the *CASP3* were significantly associated with a decreased risk for NHL (25).

The present study provides the first evidence that polymorphisms in the transcriptional regulatory region of the *CASP3* gene is associated with susceptibility to SCCHN. Because up to now no common nonsynonymous SNPs in the coding region or SNPs in the promoter region have been identified in *CASP3* and because accumulating evidence suggests that genetic polymorphisms in the promoter region may affect transcription (34), we believed that the first two exons, the first intron, and 5′-UTR likely contribute to transcription activity of *CASP3* and influence the gene expression, thus likely contributing to risk of SCCHN. Based on our results, it is likely that *CASP3* rs4647601:T allele, also presented in the risk haplotype TCG, may be functional because it was associated with risk of SCCHN in an allele dose-response fashion, particularly in younger subjects, never smokers, and never drinkers. It is possible that this T allele may have caused reduction in the apoptotic capacity of the target tissue and thus increased the potential of carcinogenesis. Nevertheless, this mechanistic speculation will have to be validated experimentally in the future.

Our findings of a significantly elevated risk, most evident in younger subjects, nonsmokers, and nondrinkers with a trend of increased risk with increased number of variant alleles, suggest that the *CASP3* SNPs may be markers for genetic susceptibility because the well-known characteristics of genetic susceptibility include an early age of onset with minimal amount of exposure to carcinogens. It is also possible that never smokers may have been exposed to passive smoking or other unknown carcinogens in the environment. Because our study is still not large enough to provide sufficient statistical power, we failed to find any evidence of interactions between the risk genotypes and exposure such as smoking and alcohol use. Additional larger studies with more detailed information on passive smoking are needed to validate our findings. However, the absence of association between the *CASP3* rs4647601:T allele and risk of SCCHN in smokers and drinkers suggests that mechanisms other than CASP3-mediated apoptosis may play a role in the carcinogenesis among these subjects. Therefore, future studies should include more SNPs of other apoptosis-related genes to unravel these mechanisms.

Our findings of the associations between the *CASP3* haplotypes and diplotypes and risk of SCCHN further strengthen those of the single-locus analyses. Although the functionality of the three *CASP3* SNPs has not been determined, it is also likely that these SNPs may be markers of untyped or unknown functional SNPs located in the same gene or genes in the nearby regions. Therefore, their haplotypes/diplotypes are more representative of the genetic variation associated with risk of SCCHN. Our additional analyses of combined primary tumor stage and lymph node metastasis status further suggest that these *CASP3* variants are more likely to be risk or susceptibility markers than disease progression markers because the risks associated with the variant diplotypes were confined to patients with early stages of the tumors without lymph node metastasis.

Given our findings, haplotypes and diplotypes containing *CASP3* rs4647601:T, rs4647602:C, and rs4647603:A alleles may be risk alleles or genotypes for SCCHN in the general population. The allele and genotype frequencies of rs4647603 (CEU: G/G = 0.7, A/G = 0.25, and A/A = 0.05 from 60 subjects and G = 0.825 and A = 0.175 from 120 chromosomes), which is the only one genotyped in the HapMap, are similar to our genotype data (G/G = 0.758, A/G = 0.225, and A/A = 0.017 from 993 control subjects and G = 0.871 and A = 0.129 from 1,986 chromosomes). However, no other studies have reported the allele and genotype frequency data on the *CASP3* rs4647601 and rs4647602. Because of unknown functionality of the *CASP3* SNPs under the study, further functional investigation of these informative SNPs of *CASP3* is warranted to better understand the mechanisms underlying the carcinogenesis in and risk association with SCCHN.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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