

Haplotypes of Two Variants in *p16* (*CDKN2/MTS-1/INK4a*) Exon 3 and Risk of Squamous Cell Carcinoma of the Head and Neck: A Case-Control Study¹

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Abstract

The frequent loss or promoter methylation of the tumor suppressor gene *p16* in head and neck cancer suggests an etiologic role of *p16* in this disease. Two adjacent polymorphisms of *p16* exon 3, C540G and C580T, were identified recently. C540G is associated with low expression of p53, and both polymorphisms are associated with tumor aggressiveness, suggesting a possible functional relevance. We hypothesized that these two polymorphisms, particularly their haplotypes, are associated with the risk of developing squamous cell carcinoma of the head and neck (SCCHN). To test this hypothesis, we conducted a hospital-based case-control study of 208 patients with SCCHN and 224 cancer-free control subjects to evaluate the association between *p16* genotypes/haplotypes and the risk of SCCHN, using a PCR-single strand conformation polymorphism-based genotyping assay. However, our results suggested that no significant differences exist in the distribution of *p16* C540G and C580T genotypes between cases and controls. For the C540G polymorphism, the CC, CG, and GG genotype frequencies were 76.9%, 22.1%, and 1.0%, respectively, in the cases, compared with 76.8%, 21.9%, and 1.3%, respectively, for the controls. For the C580T polymorphism, the CC, CT, and TT genotype frequencies were 83.6%, 15.9%, and 0.5%, respectively, in the cases, compared with 82.6%, 16.5%, and 0.9%, respectively, for the controls. The frequencies of three predominant 540C/580C, 540G/580C, and 540C/580T haplotype alleles were distributed similarly in the cases (79.6%, 12.0%, and

8.4%) and in the controls (78.6%, 12.3%, and 9.1%). None of these differences were statistically significant. We conclude that these polymorphic *p16* genotypes or haplotypes may not play a major role in the etiology of SCCHN, if any. However, our limited sample size and power call for larger studies for additional verification of our findings.

Introduction

Although tobacco smoking and alcohol consumption are major risk factors for SCCHN³ (1), only a small fraction of smokers or drinkers develop this disease, suggesting that genetic factors also play an etiologic role (2, 3). Reduced DNA repair capacity, which is likely modulated by polymorphisms in genes that participate in DNA repair (4) and cell-cycle control (5), are associated with an increased risk of SCCHN (6).

In response to DNA damage, normal cell-cycle control is arrested, allowing DNA damage to be repaired before the cell proceeds to mitosis and division (7). This G₁/S phase checkpoint of the cell cycle is regulated by several genes, including *p53* and *p16* (also known as *CDKN2/MTS-1/INK4a*) tumor suppressor genes (8). Whereas *p53* regulates the G₁/S checkpoint by transcriptional control of *CDKN1A* and *CDKN1B*, *p16* plays a pivotal role in the G₁/S transition (5) by regulating the *p53* pathway of cell-cycle control, leading to tumor suppression (9). The frequent loss or methylation of *p16* in head and neck cancer suggests that *p16* has a role in the etiology of this disease. For instance, *p16* inactivation predominantly caused by homozygous deletions and methylation is common in primary head and neck tumors (10–12), and even in the presence of a normal *p53* pathway, abnormal *p16* function is associated with SCCHN tumorigenesis (13–17).

p16 is polymorphic (18, 19), and two adjacent polymorphisms (C540G and C580T) were identified recently in the 3' untranslated region of exon 3 (GenBank accession no. L27211); furthermore, the C580T polymorphism cosegregates with the C74A polymorphism of intron 1 of *CDKN2B* (18). Because the C540G polymorphism is associated with low expression of *p53*, and both polymorphisms are associated with tumor aggressiveness (18), these two variants may have some functional relevance. Therefore, we hypothesized that these two variants, particularly their haplotypes, are over-represented in SCCHN cases compared with healthy controls. To test this hypothesis, we conducted a hospital-based case-control study of 208 patients with SCCHN and 224 cancer-free controls to evaluate the

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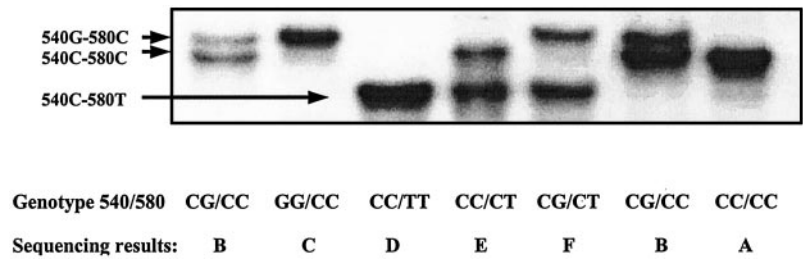
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³ The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; SSCP, single-strand conformation polymorphism; OR, odds ratio; CI, confidence interval.

Fig. 1. PCR-SSCP analysis for genotyping/haplotyping the *p16* exon 3 C540G and C580T polymorphisms. The bands labeled with letters A–F on the bottom line correspond to the genotypes designated in the upper line. Each genotype (A–F) has been confirmed by direct sequencing using both sense and antisense (not shown) primers as shown in Fig. 2.



association between *p16* genotypes/haplotypes and the risk of SCCHN using a PCR-SSCP-based genotyping assay.

Materials and Methods

Study Population. The recruitment of study subjects has been described in detail previously (5). The 208 patients with histopathologically confirmed SCCHN were recruited from the Department of Head and Neck Surgery at our institution between May 1999 and October 2000. The participation rate was ~95% among patients contacted. The 224 healthy control subjects were recruited during the same period from a local managed care organization with multiple clinics throughout the Houston metropolitan area. The participation rate among eligible controls was 73.3%. To evaluate the main effect of the *p16* polymorphisms, the control subjects were frequency matched to the cases by age (± 5 years), sex, ethnicity, and smoking status (current, former, and never). After informed consent was obtained, each subject donated 30 ml of blood, which was collected in heparinized Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and used for DNA extraction. The research protocol was approved by our Institutional Review Board.

PCR-SSCP. Genomic DNA was extracted using the Qiagen DNA blood mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. PCR-SSCP analysis was used to genotype two *p16* polymorphisms in the 3' untranslated region of exon 3. For PCR, the primers described previously (17) were used: 5'-gcc tgt ttt ctt tct gcc ctg t-3' (sense) and 5'-cga aag cgg ggt ggg ttg t-3' (antisense; confirmed by the reported sequences: GenBank accession no. L27211), which generated a 144-bp fragment. The fragment was amplified in 25 μ l of reaction mixture containing ~50 ng of genomic DNA; 6.25 pmol of each primer; 1 \times PCR buffer [50 mM KCl, 10 mM Tris HCl (pH 9.0 at 25°C) and 0.1% Triton X-100]; 1.5 mM MgCl₂; 0.2 mM each dATP, dTTP, and dGTP, 0.1 mM dCTP; and 1 μ Ci of [³²P]dCTP and 1 unit of Taq polymerase (Promega Corporation, Madison, WI). PCR was performed by incubating the fragments and reaction mixture at 95°C for 5 min; subjecting them to 28 cycles of 94°C for 30 s, 58°C for 45 s, and incubation at 72°C for 45 s; and a final elongation step at 72°C for 10 min.

For SSCP analysis, 4 μ l of PCR product was mixed with 4 μ l of loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue). This mixture was denatured at 95°C for 5 min and immediately put on ice. The mixture was loaded on the 6% mutation detection-enhancement gel (FMC BioProducts, Rockland, ME) for electrophoresis at 35 W for 5 h. After electrophoresis, gels were dried, and band images were captured by exposure of X-ray film to the gels. The bandshift patterns were then visualized by autoradiography (Fig. 1). About 10% of the samples were repeated, and the reproducibility is 100%.

Sequencing for Genotyping and Haplotyping. To determine the genotype, whole PCR products were sequenced. To determine the haplotype, the individual PCR-shifted bands were cut from the dry polyacrylamide gel as the template for additional PCR amplification by using the same primer set. The subsequent PCR products were purified with a QIAEX II Gel extraction kit (Qiagen) and sequenced by using an automated sequencer (ABI 377; PE Biosystems, Foster City, CA) with both sense and antisense PCR primers. On the basis of the bandshift patterns and results of whole PCR products sequencing, the following genotypes were identified: CC, CG, and GG of C540G and CC, CT, and TT of C580T (Fig. 2). On the basis of the sequence results of individual band, three common haplotypes were identified (Fig. 3). The wild-type haplotype allele was 540C/580C, and the two variant haplotype alleles were 540G/580C and 540C/580T (Fig. 3). We did not find the 540G/580T haplotype allele in this study population.

Statistical Analysis. The distribution of selected demographic variables, risk factors, and frequencies of *p16* variant alleles and genotypes were compared between the cases and controls by the χ^2 test. For sample size and power calculation, we modeled the frequencies of genotypes as counts in a multinomial distribution for the cases and controls. Then we used the Wilcoxon linear rank test for comparing two multinomial populations for finding the power of the tests to detect differences. These tests are asymptotically distributed as a χ^2 distribution with noncentrality parameter (20). The computation was implemented in StatXact (Version 4; Sytel Software Cooperation, Cambridge, MA) with the methods by Mehta *et al.* (21). Those subjects who had smoked <100 cigarettes in their lifetimes were defined as ever smokers, those who had quit smoking more than 1 year previously were defined as former smokers, and the others as current smokers. Those who drunk alcoholic beverages at least once a week for >1 year in previous years were defined as ever users of alcohol; of these, those who had quit drinking for more than 1 year previously were defined as former drinkers, and the others as current drinkers. The crude ORs and their 95% CIs for risk associated with each genotype and haplotype were calculated by logistic regression analysis. All of the statistical analyses were performed with Statistical Analysis System software (Version 6.01; SAS Institute Inc., Cary, NC).

Results

The analysis included 208 patients with SCCHN and 224 healthy control subjects. The characteristics of the subjects are summarized in Table 1. No differences were found in the distributions of age, sex, smoking, and alcohol use between the cases and controls, suggesting that the frequency matching was adequate. The distributions of genotype and allele frequencies of the two polymorphisms between the cases and controls are

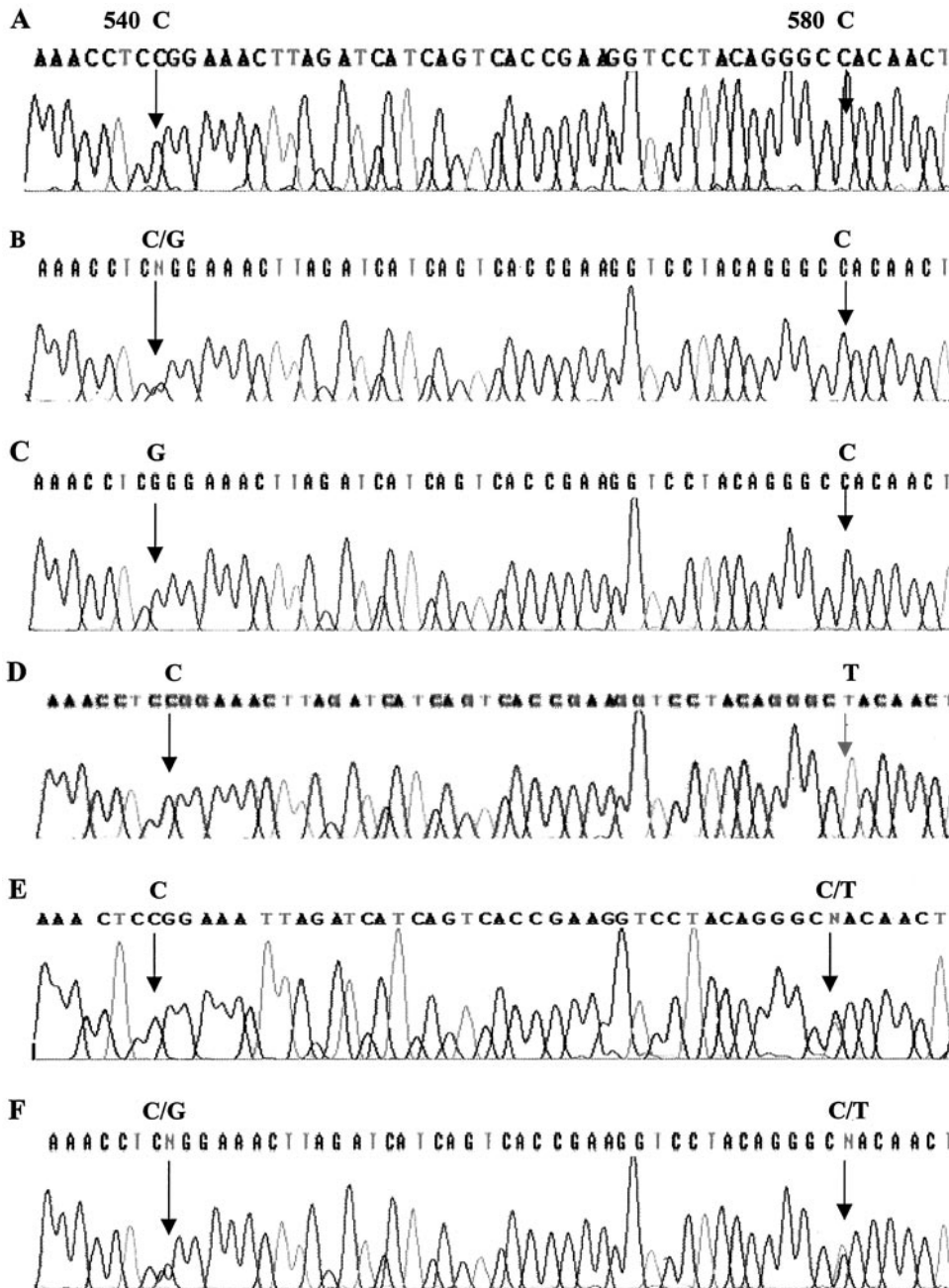


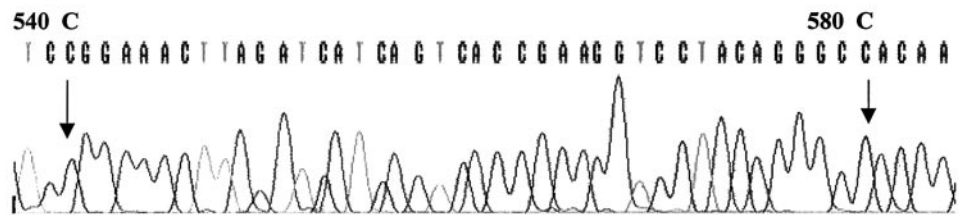
Fig. 2. Sequencing analysis for genotyping the *p16* C540G and C580T polymorphisms (using whole PCR products containing one or more bands as shown in Fig. 1). The detected six genotypes labeled by letters A–F correspond to the PCR-SSCP results shown in Fig. 1.

summarized in Table 2. For the C540G polymorphism, the variant 540G allele frequency was similar between cases and controls (0.120 versus 0.123, respectively). The frequencies of the CC, CG, and GG genotypes were 76.9%, 22.1%, and 1.0%, respectively, in the cases, which were similar to those in the controls (76.8%, 21.9%, and 1.3%, respectively). Likewise, for the C580T polymorphism, the variant 580T allele frequency was also similar for both groups (0.084 versus 0.092). Frequencies of CC, CT, and TT genotypes were 83.6%, 15.9%, and 0.5%, respectively, in the cases, which were similar to those in the controls (82.6%, 16.5%, and 0.9%, respectively). The genotype distribution of the variant C540G and C580T in the controls was in agreement with the Hardy-Weinberg equilibrium

($P = 0.994$ for C540G and $P = 1.000$ for C580T), suggesting that there was no influence from control selection. However, none of the variant genotypes were associated with risk of SCCHN (Table 2).

The analyses of the haplotype genotypes and haplotype allele frequencies are summarized in Table 3. Theoretically, 10 haplotype genotypes are possible (*i.e.*, CC/CC, CC/CT, CC/GC, CC/GT, CT/GC, CT/CT, CT/GT, GC/GC, GC/GT, and GT/GT), but only 6 haplotype genotypes were identified in this study population. No difference was observed in the distribution of the haplotype genotypes between cases and controls. The frequencies of the three predominant 540C/580C, 540G/580C, and 540C/580T haplotype alleles were 79.6%, 12.0%,

CC haplotype allele



GC haplotype allele



CT haplotype allele



Fig. 3. Sequencing analysis for haplotyping the *p16* C540G and C580T alleles (using each of individual gel bands as shown in Fig. 1). Three haplotype alleles, *i.e.*, CC (540C/580C), GC (540G/580C), and CT (540C/580T), were identified, but the GT (540G/580T) was not identified in this study population.

Table 1 Distribution of selected variables in SCCHN patients and controls

Variable	Cases (<i>n</i> = 208)		Controls (<i>n</i> = 224)		<i>P</i> ^a
	Number	%	Number	%	
Age (years)					0.613
≤45	36	17.3	36	16.1	
46–55	61	29.3	76	34.0	
56–65	54	26.0	48	21.4	
>65	57	27.4	64	28.6	
Sex					0.573
Male	152	73.1	169	75.5	
Female	56	26.9	55	24.5	
Smoking status					0.793
Never	61	29.3	70	31.2	
Former	94	45.2	94	42.0	
Current	53	25.5	60	26.8	
Alcohol use					0.340
Never	96	46.2	119	53.1	
Former	68	32.7	62	27.7	
Current	44	21.1	43	19.2	

^a Two-sided χ^2 test.

and 8.4%, respectively, in the cases, and 78.6%, 12.3%, and 9.1%, respectively, in the controls. These differences were not statistically significant. Additional logistic regression analysis using the 540C-580C/540C-580C wild-type haplotype allele as the reference revealed no statistically significant associations between either the C540G and C580T polymorphisms or the haplotypes and the risk of SCCHN. By a two-sided test with a

0.05 significance level, our study had an 80% power to detect a minimal OR of 2.0, assuming a risk, or a maximal of 0.5, assuming a protective effect, with a difference in the variant genotype frequencies of 12% between the cases and the controls based on the observed 23.2% and 17.4% for the 540G (CG/GG) and 580T (CT/TT) genotypes (Table 2), respectively, in the controls.

Discussion

In this study, we tested the hypothesis that two *p16* exon 3 polymorphisms, particularly their haplotypes, are associated with the risk of developing SCCHN. We report that the 540G and 580T allele frequencies were relatively low (0.123 and 0.092, respectively) and that the variant homozygous genotypes of these two polymorphisms (540GG and 580TT) were rare (1.3% and 0.9%, respectively) in 224 healthy control subjects. The 540G and 580T allele frequencies in the cases were 0.120 and 0.084, respectively, compared with the reported frequencies of 0.10 and 0.13, respectively, in 48 metastatic melanoma patients (18), 0.18 and 0.13, respectively, in another study of 31 melanoma patients (22), and 0.03 and 0.03, respectively, in 20 esophageal squamous cell carcinoma patients (10). However, we found no evidence for an association between these variant genotypes or their haplotype genotypes and the risk of developing SCCHN. To the best of our knowledge, this is the first case-control study of SCCHN that has investigated the role of these two polymorphisms in the etiology of SCCHN.

In normal cells, *p16* functions to negatively regulate the cell cycle by inhibiting cyclin-dependent kinases 4 and 6, and

Table 2 Distribution and logistic regression analysis of the *p16* exon 3 genotypes and allele frequencies in SCCHN patients and controls

Genotype	Cases (n = 208)		Controls (n = 224)		Crude OR (95% CI)	Adjusted ^a OR (95% CI)
	Number	%	Number	%		
C540G						
CC	160	76.9	172	76.8	1.00	1.00
CG	46	22.1	49	21.9	1.01 (0.64–1.59)	1.01 (0.64–1.61)
GG	2	1.0	3	1.3	0.72 (0.12–4.35)	0.74 (0.12–4.57)
CG/GG	48	23.1	52	23.2	0.99 (0.63–1.55)	1.00 (0.64–1.57)
G allele frequency ^b		0.120		0.123		
C580T						
CC	174	83.6	185	82.6	1.00	1.00
CT	33	15.9	37	16.5	0.95 (0.57–1.58)	0.97 (0.58–1.64)
TT	1	0.5	2	0.9	0.53 (0.05–5.92)	0.49 (0.04–5.49)
CT/TT	34	16.4	39	17.4	0.93 (0.56–1.54)	0.94 (0.57–1.58)
T allele frequency ^b		0.084		0.092		

^a Adjusted for age, sex, smoking status, and alcohol use.

^b Two-sided χ^2 test: for 540G allele frequency, $P = 0.976$; for C540G genotype distribution, $P = 0.934$; for 580T allele frequency, $P = 0.768$; and for C580T genotype distribution, $P = 0.858$.

Table 3 Distribution of the *p16* exon 3 haplotype genotype and allele frequency in SCCHN patients and controls

<i>p16</i> Haplotype	Cases (n = 208)		Controls (n = 224)	
	Number	%	Number	%
Genotypes ^a				
540C-580C/540C-580C	134	64.4	136	60.7
540C-580C/540C-580T	25	12.0	34	15.2
540C-580C/540G-580C	38	18.3	46	20.5
540C-580T/540C-580C	8	3.9	3	1.3
540C-580T/540C-580T	1	0.5	2	0.9
540G-580C/540G-580C	2	1.2	3	1.3
540C-580C/540G-580T	0	0	0	0
540G-580C/540G-580T	0	0	0	0
540C-580T/540G-580T	0	0	0	0
540G-580T/540G-580T	0	0	0	0
Allele frequencies ^b				
CTCCGG GGCCAC	331	79.6	352	78.6
CTC_GGG GG_C_CAC	50	12.0	55	12.3
CTCCGG GGCTAC	35	8.4	41	9.1
CTC_GGG GGCTAC	0	0	0	0
Total alleles	416	100	448	100

^a For the *p16* C540G/C580T haplotype genotype distribution: $\chi^2 = 4.369$, df = 5, $P = 0.574$.

^b For the haplotype allele distribution: $\chi^2 = 0.173$, df = 2, $P = 0.917$.

subsequently blocking of cyclin-dependent phosphorylation of Rb (22). In tumors, *p16* is frequently deleted, mutated, or methylated (10–17), and loss of p16 functions leads to deregulation of the suppressive block of the G₁/S transition of both p53 and Rb in the cell cycle (23). Although the two polymorphisms investigated in this study were reportedly associated with lower expression of p53 proteins in tumor cells and with tumor aggressiveness (18), their functional relevance in normal tissues remains unknown. Our results suggest that these two *p16* polymorphisms themselves may not result in abnormal cell-cycle control and, therefore, do not contribute to susceptibility to SCCHN. Because of the importance of *p16* in regulating cell-cycle control, additional investigation of functionally relevant *p16* polymorphisms and their role in the etiology of cancer is warranted.

In summary, we have developed a method that allows haplotyping for two adjacent *p16* variants, C540G and C580T. However, our results did not support our *priori* hypothesis that these two variants are over-represented in

SCCHN cases. Nevertheless, our established *p16* haplotyping method should be useful for future studies to investigate genetic susceptibility to other cancers or germ-line mutation carriers in which the *p16* haplotype may act as an effect modifier similar to the *CCND1* polymorphism in *hMLH1* and *hMSH2* mutation carriers (24, 25).

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