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A novel functional variant (−842G>C) in the *PIN1* promoter contributes to decreased risk of squamous cell carcinoma of the head and neck by diminishing the promoter activity

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PIN1, a new peptidyl-prolyl *cis/trans* isomerase, regulates the conformation of Pro-directed phosphorylation sites, revealing a new postphosphorylation regulatory mechanism. PIN1-induced conformational changes potentiate multiple oncogenic signaling pathways, and PIN1 overexpression is reported as a prevalent and specific event in human cancers. In this study, we tested the hypothesis that common polymorphisms in the coding and promoter regions of *PIN1* are associated with risk of squamous cell carcinoma of the head and neck (SCCHN). We genotyped three selected *PIN1* polymorphisms (−842G>C, −667T>C and Gln33Gln) in a hospital-based case–control study of 1006 patients with SCCHN and 1007 cancer-free control subjects. We found that the −842C variant genotypes were associated with decreased risk for SCCHN [Odds Ratio (OR) = 0.74; 95% confidence interval (CI) = 0.59–0.93 for the CG genotype, OR = 0.82; 95% CI = 0.34–2.01 for the CC genotype and OR = 0.74; 95% CI = 0.59–0.93 for CG+CC genotypes, compared with the GG genotype]. However, no altered risks were observed for −667T>C and Gln33Gln polymorphisms. Further experiments of the reporter gene expression driven by the allelic *PIN1* promoter showed that the −842G allele had a higher activity than that driven by the −842C allele, suggesting that the −842C allele was associated with a reduced transcriptional activity, a finding consistent with a reduced risk observed in the case–control analysis. Large prospective studies of diverse ethnic groups and diverse cancer sites are warranted to validate our findings.

Introduction

Pro-directed phosphorylation is an important signaling mechanism controlling diverse cellular processes, including cell cycle progression, transcriptional regulation, RNA processing and cell proliferation and differentiation (1,2). The mechanisms controlling Pro-directed phosphorylation are important both physiologically and pathologically (3) in regulating the functions of a subset of Ser/Thr-Pro-containing proteins (1,4), and the deregulation of this event can result in cell transformation and oncogenesis (1). Peptidyl-prolyl *cis/trans* isomerase, PIN1, specifically regulates the conformation of Pro-directed phosphorylation sites, revealing a new postphosphorylation regulatory mechanism (5,6).

PIN1 substrates include many essential cell cycle regulators as well as oncogenic and tumor suppressor proteins, such as cyclin D1, Cdc25, c-Jun, β-catenin, Bcl-2, p73 and p53 (1,3). Therefore, PIN1-induced conformational changes may function as a critical catalyst that potentiates multiple oncogenic signaling pathways during cancer development (4). Studies have shown that PIN1 expression has an oncogenic role in some common cancers, such as prostate (7), breast (8,9) and oral squamous cell carcinoma (10), and its overexpression is a prevalent and specific event in human cancers (11,12). In contrast, inhibition of PIN1 in cancer cells can trigger apoptosis or suppress the transformed phenotype (5,12,13). Furthermore, PIN1 is recently identified as a novel negative forkhead box O regulator, in response to cellular stress, that regulates p27(kip1) (14). Therefore, PIN1 may be involved in DNA damage and repair and thus in carcinogenesis.

Several common polymorphisms have been identified in the coding and promoter regions of *PIN1* (http://www.ncbi.nlm.nih.gov/SNP/ and ref. 15), including two variants in the promoter region: rs2233678 (G>C) at nucleotide −842 and rs2233679 (T>C) at nucleotide −667 and one synonymous change (Gln33Gln; G>A, rs2233682) in the coding region (Figure 1A). Recently, one study investigated the roles of *PIN1* rs2233678 −842G>C single-nucleotide polymorphism (SNP) and rs2233679 −667C>T SNPs in the etiology of hepatocellular carcinoma (HCC) and found that the −667 T allele, but not −842C, may contribute to the risk of HCC (16). We previously investigated the roles of genetic variants of several genes, which encode cyclin D1, p73 and p53 proteins that are PIN1 substrates, in the etiology of head and neck cancer (17–19). In the present study, we evaluated both the association between three reported SNPs and cancer risk as well as the promoter activity that may be mediated by potentially functional *PIN1* variants.

Materials and methods

Study population

The recruitment of study subjects was described previously (20). Briefly, the study population included 1010 non-Hispanic white patients with newly diagnosed squamous cell carcinoma of the head and neck (SCCHN) and 1010 cancer-free frequency-matched control subjects from among hospital visitors recruited between May 1995 and September 2005. After written informed consent was obtained, each eligible subject provided a venous blood sample of ∼30 ml and information on environmental exposure history including tobacco and alcohol use. Our institutional review board approved the research protocol.

Genotyping analysis

We first genotyped the two common (minor allele frequency >0.05) SNPs (*PIN1* −842G>C, rs2233678 and −667T>C, rs2233679) in the promoter region and one common synonymous SNP in exon 2 (Gln33Gln (G>A; rs2233682) selected among 73 SNPs reported in the dbSNP database because no non-synonymous SNPs have been reported for the *PIN1* gene (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?chooseRs=all&go=Go&locusId=5300).

Since the rs2233678 and rs2233679 are close in distance, we used one pair of primers [5′-CGGGCTCTGCAGACTCTATT-3′ (forward) and 5′-AAATTTGGCTCCTCCATCCT-3′ (reverse)] and two different enzymes to identify the respective genotypes [BanII (New England BioLabs, Beverly, MA) was used for rs2233678 and SacI (New England BioLabs) was used for rs2233679], and we used the pair of primers 5′-GGAGCACAACCCTAGCTGAA-3′ (forward) and 5′-GGCTGTGCTTCACCAGCA-3′ (reverse) and enzyme BsrI (New England BioLabs) for the identification of rs2233682, using the polymerase chain reaction–restriction fragment length polymorphism method. The amplified fragments were then digested, separated in 3% MetaPhor agarose gel and confirmed by direct sequencing, and the genotypes were determined as shown in Figure 1A. About 10% of the samples were randomly selected for repeated assays and the results were 100% concordant. Because the two promoter SNPs have not been genotyped in the HapMap database, we then genotyped additional four common known *PIN1* tagging SNPs (rs4804461, rs2287838, rs2010457 and rs889162) obtained

Abbreviations: CI, confidence interval; HCC, hepatocellular carcinoma; LD, linkage disequilibrium; OR, odds ratio; SCCHN, squamous cell carcinoma of the head and neck; SNP, single-nucleotide polymorphism.

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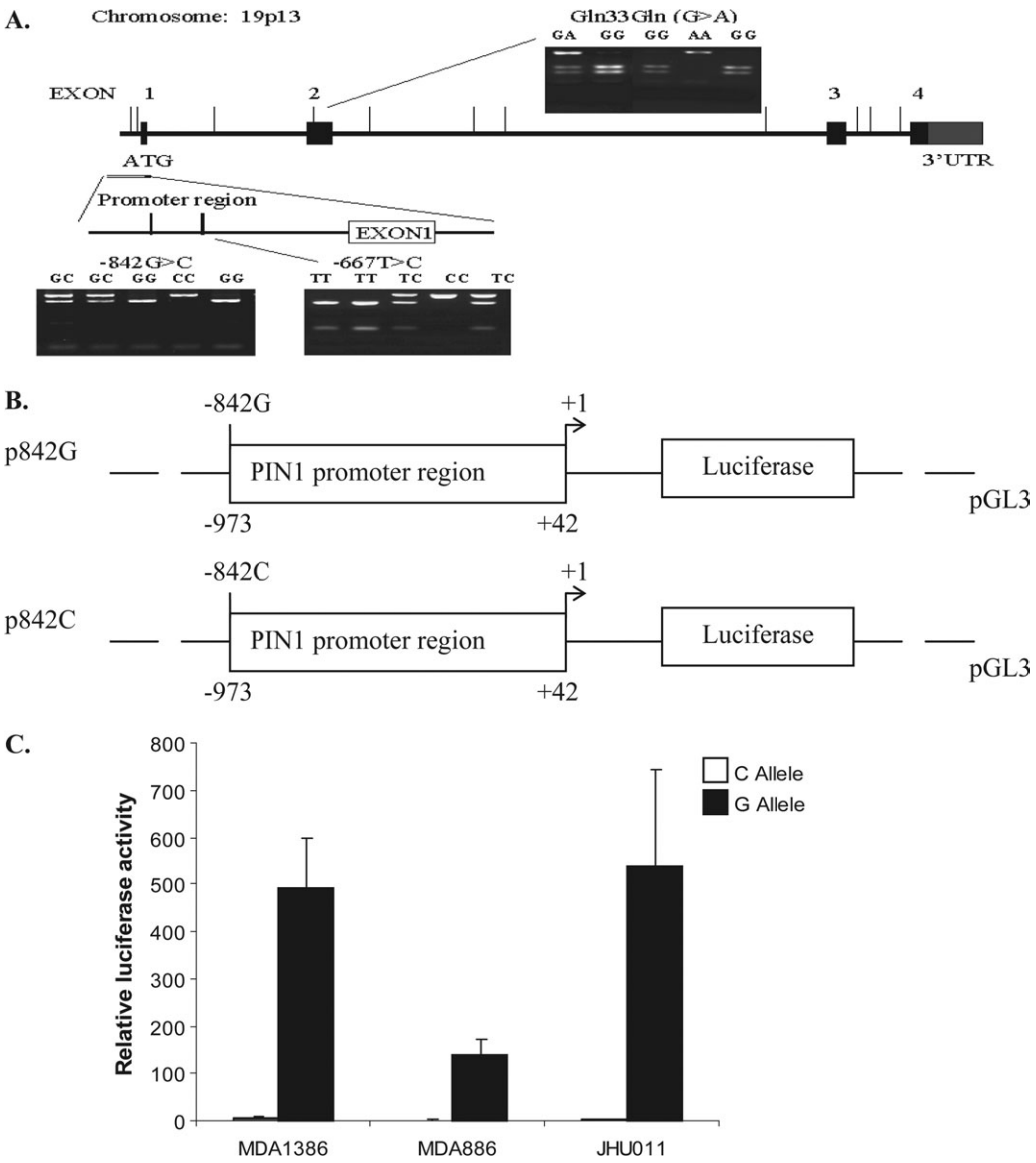

Fig. 1. *PIN1* gene structure, reporter gene constructs for the *PIN1* promoter and luciferase expression of the constructed promoter in different cell lines. (**A**) Genomic structure, locations and genotypes of three selected *PIN1* SNPs. (**B**) Schematic drawing of the reporter gene constructs containing a 1016 bp *PIN1* promoter region; the only difference between the two constructs was a G or C at the −842 polymorphic site. (**C**) Luciferase activity of the two *PIN1* promoter constructs in three head and neck cancer cell lines: MDA1386ln, MDA886 and JHU011. Fold increase was measured as the activities of the reporter gene constructs relative to that of the empty pGL3 basic vector using the data (mean ± SD) from three independent transfection experiments: $P < 0.001$ for all comparisons of each cell line between the activities of the reporter gene constructs.

from the HapMap database in 110 randomly selected control subjects. Estimates of pairwise linkage disequilibrium (LD) of these seven SNPs based on the r^2 statistics were obtained using HAPLOVIEW version 4.1. The LD output showed a low LD between the three selected SNPs and the four tagging SNPs (data no shown), suggesting the three selected SNPs were unique and informative, which we genotyped for all subjects included in this case–control study.

Construction of reporter plasmids

The *G* and *C* allelic reporter constructs were prepared by amplifying the 1016 bp *PIN1* promoter region (from −973 to +42 relative to the translation start site) from subjects homozygous (*GG* and *CC*) for the −842 variant using the primers 5′-aa***ggtacc***gcaccctttgctgtcagtgtct-3′ (forward) and 5′-aa***gctagc***cggctcatgcgcttctcc-3′ (reverse), including the KpnI and NheI restriction sites. The amplicons and the pGL3 basic vector (Promega, Madison, WI) were cleaved by using KpnI and NheI (New England BioLabs) and then ligated by T4 DNA ligase (New England BioLabs). The p842G and p842C constructs were sequenced to confirm the orientation and integrity of each insert (Figure 1B).

Transient transfection and luciferase assays

The human head and neck carcinoma cell lines, MDA-1386Ln, MDA-886 and JHU011, were cultured either in Dulbecco's modified Eagle's medium/1×, Dulbecco's modified Eagle's medium/F12 or RPMI 1640 medium with 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO) at 37°C in 5% CO_2. The cultured cells were then transiently transfected with 1.5 μg of p842T or p842C constructs. FuGENE 6 (Roche Applied Science, Indianapolis, IN) was used to transfect these cell lines. The pGL3 basic vector without the insert was used as a negative assay control. The pRL-TK vector (Promega) was cotransfected as an internal control for transfection efficiency. Luciferase activity was quantified by a Dual-Luciferase Reporter Assay System (Promega), and relative luciferase activity was calculated according to the manufacturer's instructions using a luminometer (TD-20/20 DLReasy, Promega). Promoter activity was calculated for each construct as a ratio of the luciferase activity to that of the pGL3 basic vector (Figure 1C).

Statistical analysis

Differences in the selected demographic variables, smoking and drinking status and frequencies of the *PIN1* genotypes between the cases and controls were

evaluated by using the χ^2 test. The associations between *PIN1* variants and SCCHN risk were estimated by computing the odds ratios (ORs) and 95% confidence intervals (CIs) from both univariate and multivariate logistic regression analyses. We applied the PROC HAPLOTYPE procedure in SAS/Genetics software to infer haplotype frequencies between the two promoter variants based on their observed genotypes. All the statistical analyses were performed with Statistical Analysis System software (v.9.1.3; SAS Institute, Cary, NC).

Results

DNA quality or quantity was insufficient for the *PIN1* genotyping in seven subjects (four cases and three controls); thus, the final analysis included 1006 cases and 1007 controls. Because of frequency matching, all subjects were non-Hispanic whites with similar mean ages (56.9 ± 11.3 years and 57.4 ± 11.4 years for cases and controls, respectively) and sex ratios (22.9 and 22.8% of women for the cases and controls, respectively). However, the cases were reportedly more probable than the controls to be smokers (current smokers: 34.3 versus 23.3%; former smokers: 39.3 versus 44.3%) and more drinkers (current drinkers: 50.3 versus 42.7%; former drinkers: 24.1 versus 24.6%; $P < 0.001$ for smoking status and $P = 0.001$ for drinking status). Of the 1006 cases, 282 (28.0%) had cancers of the oral cavity, 537 (53.4%) of the oropharynx (including 44 hypopharynx) and 187 (15.6%) of the larynx.

The observed genotype frequencies for these three SNPs were all in Hardy–Weinberg equilibrium in the control subjects ($P = 0.64$ for −842G>C, $P = 0.08$ for −667T>C and $P = 0.60$ for Gln33Gln). Only the distribution of *PIN1* −842G>C genotype frequencies between the cases and the controls was significantly different ($P = 0.039$) (Table I). Compared with the −842GG genotype, the −842GC heterozygote was associated with a significantly decreased SCCHN risk (OR = 0.74; 95% CI = 0.59–0.93), and the −842CC

Table I. Distribution of *PIN1* polymorphisms and logistic regression analysis for association with risk of SCCHN

Genotypes	Cases ($N = 1006$), *n* (%)	Controls ($N = 1007$), *n* (%)	P^a	Crude OR (95% CI)	Adjusted OR (95% CI)[b]
PIN1 −842G>C					
GG	838 (83.3)	794 (78.8)	0.039	1.00 (ref.)	1.00 (ref.)
GC	159 (15.8)	202 (20.1)		**0.75 (0.59–0.94)**	**0.74 (0.59–0.93)**
CC	9 (0.9)	11 (1.1)		0.78 (0.32–1.88)	0.82 (0.34–2.01)
GC + CC	168 (16.7)	213 (21.2)		**0.75 (0.60–0.94)**	**0.74 (0.59–0.93)**
C allele	0.088	0.111	0.014		
PIN1 −667 T>C					
TT	427 (42.5)	443 (44.0)	0.693	1.00 (ref.)	1.00 (ref.)
TC	474 (47.1)	468 (46.5)		1.05 (0.87–1.26)	1.04 (0.87–1.25)
CC	105 (10.4)	96 (9.5)		1.14 (0.84–1.54)	1.16 (0.85–1.57)
TC + CC	579 (57.5)	574 (56.0)		1.07 (0.89–1.27)	1.06 (0.89–1.27)
C allele	0.340	0.328	0.410		
PIN1 Gln33Gln, G>A					
GG	944 (93.8)	934 (92.7)		1.00 (ref.)	1.00 (ref.)
GA	61 (6.1)	71 (7.1)	0.565	0.85 (0.60–1.21)	0.89 (0.62–1.27)
AA	1 (0.1)	2 (0.2)		0.50 (0.05–5.47)	0.60 (0.05–6.77)
GA + AA	62 (6.2)	73 (7.3)		0.84 (0.59–1.19)	0.88 (0.62–1.25)
A allele	0.031	0.037	0.301		

[a]Two-sided $\chi 2$ test for either genotype distribution or allele frequency.
[b]Adjusted for age, sex, smoking status, and alcohol use in a logistic regression model.

Table II. Stratification analysis of the *PIN1* −842G>C genotypes by selected variables in SCCHN cases and controls

	Cases ($N = 1006$)		Controls ($N = 1007$)		Crude OR (95% CI)	Adjusted OR (95% CI)[a]
	GG, *n* (%)	GC + CC, *n* (%)	GG, *n* (%)	GC + CC, *n* (%)	GC + CC versus GG	GC + CC versus GG
Age (years)						
<50	205 (81.0)	48 (19.0)	203 (79.6)	52 (20.4)	0.91 (0.59–1.42)	0.89 (0.57–1.38)
50–60	312 (83.0)	64 (17.0)	262 (77.7)	75 (22.3)	0.72 (0.49–1.04)	0.73 (0.50–1.06)
>60	321 (85.1)	56 (14.9)	329 (79.3)	86 (20.7)	**0.67 (0.46–0.97)**	**0.67 (0.46–0.97)**
Sex						
Female	198 (86.1)	32 (13.9)	173 (75.2)	57 (24.8)	**0.49 (0.30–0.79)**	**0.47 (0.29–0.76)**
Male	640 (82.5)	136 (17.5)	621 (79.9)	156 (20.1)	0.85 (0.66–1.09)	0.84 (0.65–1.09)
Smoking status						
Never	226 (85.0)	40 (15.0)	266 (81.6)	60 (18.4)	0.79 (0.51–1.22)	0.79 (0.51–1.22)
Former	334 (84.6)	61 (15.4)	354 (79.4)	92 (20.6)	0.70 (0.49–1.00)	0.71 (0.50–1.02)
Current	278 (80.6)	67 (19.4)	174 (74.0)	61 (26.0)	0.69 (0.46–1.02)	0.72 (0.48–1.07)
Drinking status						
Never	214 (83.3)	43 (16.7)	255 (77.5)	74 (22.5)	0.69 (0.46–1.05)	0.68 (0.45–1.04)
Former	201 (82.7)	42 (17.3)	194 (78.2)	54 (21.8)	0.75 (0.48–1.18)	0.76 (0.48–1.20)
Current	423 (83.6)	83 (16.4)	345 (80.2)	85 (19.8)	0.80 (0.57–1.11)	0.78 (0.56–1.10)
Tumor site						
Oral cavity	233 (82.6)	49 (17.4)			0.78 (0.56–1.11)	0.77 (0.55–1.10)
Pharynx[b]	452 (84.2)	85 (15.8)	794 (78.8)	213 (21.2)	**0.70 (0.53–0.93)**	**0.71 (0.53–0.94)**
Larynx	153 (81.8)	34 (18.2)			0.83 (0.56–1.24)	0.78 (0.52–1.18)

[a]ORs were adjusted for age, sex, smoking status and alcohol use in a logistic regression model.
[b]Including oropharynx and hypopharynx.

homozygote with a non-significantly decreased SCCHN risk (OR = 0.82; 95% CI = 0.34–2.01). Because the −842CC genotype was relatively rare in this study population, we combined it with the −842GC genotype, assuming a dominant allele effect hereafter (Table I). However, no overall associations were observed between the other two variants, −667T>C and Gln33Gln, and SCCHN risk (Table I). In the stratified analysis with the −842G>C SNP (Table II), the decreased risk associated with the variant genotypes was slightly more evident among older subjects (OR = 0.67; 95% CI = 0.46–0.97), women (OR = 0.47; 95% CI = 0.29–0.76) and patients with pharyngeal cancers (OR = 0.71; 95% CI = 0.53–0.94) but neither a difference in the stratum-related ORs nor any evidence for a significant gene–environment interaction was found (data not shown).

The LD analysis showed that the LD between two loci in the promoter region was relatively high ($D' = 0.825$ and $r^2 = 0.175$) but both were not in LD with the locus in the coding region ($D' = 0.173$, $r^2 = 0.0001$ for −842G>C and Gln33Gln; $D' = 0.219$, $r^2 = 0.0042$ for −667T>C and Gln33Gln; data not shown). Overall, four possible hapolotypes were obtained, and their distribution between the cases and controls was significantly different ($P = 0.00002$). Specifically, compared with the most common −842G–667T haplotype, only the rare −842C–667T haplotype was associated with a significantly decreased risk for SCCHN (OR = 0.003; 95% CI = 0.001–0.16; $P = 0.005$; Table III).

To directly determine an allele-specific effect of the *PIN1* −842G>C variants on the promoter activity, two luciferase reporter gene constructs were generated, which contained 1016 bp of the *PIN1* promoter region with a G or C at the −842 polymorphic site (Figure 1B). As shown in Figure 1C, reporter gene expressions driven by the −842G allelic *PIN1* promoter were much higher than those driven by the variant −842C allelic *PIN1* promoter in three head and neck cancer cell lines tested. The highest expression level was observed in JHU011 and the lowest in MDA886.

Discussion

In this study, we found that the variant −842C genotypes were associated with a decreased risk of SCCHN, a finding consistent with the diminished *PIN1* promoter activity of the variant −842C allele in the report gene expression experiments. To the best of our knowledge, this is the first report on genetic variants in *PIN1* and cancer susceptibility. Because the −842G>C variant is relatively common in the general population, our findings may have important public health relevance.

PIN1 is not an oncogene itself, because it serves only as an indispensable translator and amplifier of oncogenic signal transduction, and the fulfillment of its functions depends on the presence of other oncogenes, such as *Ras* or *Neu* (12). This is consistent with the observed high *PIN1* allelic expression in head and neck cancer cells in the present study because the Ras overexpression is a common event in cancer (21). It was reported that *PIN1*-knockout mice developed normally at a young age but exhibited age-dependent proliferative disorders in specific tissues (22). Therefore, altered basal *PIN1* transcript levels may be associated with some age-related diseases, such as Alzheimer's disease and cancer (15). It is probably that the age-related protective effect of the variant −842C allele may be responsible for the observed reduction in SCCHN risk in the older subjects in this study population.

Functional analysis of the *PIN1* −842G>C polymorphism suggested that the association of the variant with SCCHN risk might be attributed to diminished expression of the −842C allele because subjects carrying the −842C allele showed reduced levels of the PIN1 protein in their peripheral mononuclear cells (15). Although the −667C variant was in LD with the apparently causal −842C variant, our haplotype analysis further showed that the effect was mainly due to the −842C variant, and a previous study also found that the −667C variant was not associated with protein levels or disease risk (15). It has been reported that PIN1 may operate as a cell cycle molecular timer and loss of PIN1 may have a protective effect on cancer risk (23). Our expression experiments of the −842G>C variants suggest that the change from G to C may cause loss of the known gene-binding site that may regulate the PIN1 expression. However, this speculation needs to be tested in additional mechanistic studies in the future.

Genetic polymorphisms often vary between ethnic groups. A recent Italian study investigated the association between *PIN1* rs2233678 −842G>C and rs2233679 −667T>C SNPs and risk of HCC in 228 patients and 250 controls (16) because PIN1 was preferentially overexpressed in hepatitis B virus-related tumors (24). In that study, the −842G>C SNP did not show any differences in allele and genotype frequencies between HCC cases and controls, whereas the −667T>C SNP had an over presentation of the T allele in the HCC cases and in the controls; in their 250 controls, genotype frequencies were 81% for GG, 16% for CG and 3% for CC of G−842C and 42% for TT, 45% for CT and 13% for CC of −667T>C (16). The discrepancy between this study and ours in terms of the alleles involved in the etiology of different cancer types may result from cancer-specific exposure, different study sizes or ethnic admixture that contributed to the difference in the genotype frequency distribution. It is interesting that our data showed that the reduction of risk associated with the −842C allele was more pronounced in patients with pharyngeal cancer, a tumor site most probably to be associated with infection of human papilloma virus (25). Therefore, the expression of PIN1 in tumor tissues of pharyngeal cancer should be explored in future studies.

However, in a recently published report of a larger study on *PIN1* polymorphisms and risk for Alzheimer's disease in a French population, the −842G>C genotype frequencies in 655 healthy control subjects were 79% for GG, 20% for CG and 1% for CC (26), which were quite similar to those in our non-Hispanic white population (78.8% for GG, 20.1% for CG and 1.1% for CC). Likewise, for the −667T>C variant, the genotype frequencies in the 655 control subjects (47% for TT, 42% for CT and 11% for CC) (26) were also comparable with our results (44.0% for TT, 46.5% for CT and 9.5% for CC) in the 1007 controls. Our larger sample size provided much stable estimates for the allele and genotype frequencies of these SNPs.

Although our finding of an association between SCCHN risk and the −842C variant that has diminished transcriptional activity is novel, this study had some limitations. First, we did not have the opportunity to examine *PIN1* messenger RNA levels in target tissues with different −842G>C genotypes in the study subjects, and we did not know the exact mechanism of the regulation of the −842G>C variant on the *PIN1* transcript activity, though the roles of PIN1 in cell cycle and cancer are well recognized (26). Further mechanistic studies of the different −842G>C variants are warranted. It remained unclear why the risk was more pronounced in women or in those with pharyngeal cancer, although these could be chance findings due to smaller sample sizes in the stratified analyses. Because our study was hospital-based and the control subjects may not be representative of the general population, our findings warrant further validation in large population-based perspective studies with different ethnic populations.

Table III. *PIN1* −842G>C and −667T>C haplotypes and SCCHN risk

Haplotypes	Cases (2012 alleles), *n* (%)	Controls (2014 alleles), *n* (%)	OR (95% CI)[a]	*P*
−842G–667T	1326 (65.9)	1328 (65.9)	1.00 (Ref.)	Ref.
−842G–667C	509 (25.3)	462 (23.0)	1.23 (0.90–1.67)	0.190
−842C–667C	175 (8.7)	198 (9.8)	0.81 (0.52–1.27)	0.360
−842C–667T	2 (0.1)	26 (1.3)	**0.003 (0.001–0.16)**	**0.005**
		$P = 0.00002$[b]		

[a]Adjusted for age, sex, smoking status, and alcohol use.
[b]Global test.

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