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Functional repeats (TGYCC)n in the p53-inducible gene 3 (*PIG3*) promoter and susceptibility to squamous cell carcinoma of the head and neck

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A polymorphic pentanucleotide microsatellite sequence (TGYCC)n within the p53-inducible gene 3 (*PIG3*) promoter is correlated with the extent of transcriptional activation by p53 and thought to modulate susceptibility to cancer. Using a PCR–silver staining-based single-strand conformation assay, we visualized variant genotypes of the *PIG3* promoter (TGYCC)n motif in a subset of 100 subjects for each of four ethnic groups: non-Hispanic whites, African Americans, Hispanic Americans and Native Chinese. We found that *PIG3* $(TGYCC)_{15}$ was the most common allele but less frequent in non-Hispanic whites (0.660) than in Chinese (0.785) ($P = 0.016$). In an additional study of 616 patients with squamous cell carcinoma of the head and neck (SCCHN) and 623 cancer-free controls in a non-Hispanic white population, we found that compared with those who were *PIG3* $(TGYCC)_{15}$ homozygotes, subjects without the *PIG3* $(TGYCC)_{15}$ allele had a significantly increased SCCHN risk [adjusted odds ratio (OR) = 1.34; 95% CI = 1.04–1.73 for heterozygotes and OR = 1.69; 95% CI = 1.18–2.44 for variant homozygotes] in an allele-dose response manner ($P = 0.002$). Consistently, subsequent luciferase reporter assay revealed that the wild-type $(TGYCC)_{15}$ allele had the highest p53-mediated transcriptional activity, compared with the other (TGYCC)n motifs. Our data suggest that the *PIG3* variant polymorphic repeats alleles other than $(TGYCC)_{15}$ may affect p53 binding and thus may be a marker for susceptibility to SCCHN, but our findings need to be validated in larger studies.

Introduction

Many genes have been identified as the p53-targeted genes that can be divided into several groups according to their ability to mediate a specific p53 function, such as inhibition of cell growth, DNA repair, activation of apoptosis or regulation of angiogenesis (1–3). Therefore, the major biological responses triggered by p53, including cell cycle arrest, senescence, differentiation and apoptosis, are both intrinsic and extrinsic to the cell (1,2,4).

The p53-inducible gene 3 (*PIG3*) involved in the p53-initiated apoptosis is one of the p53 downstream genes that have a p53-response element (2,5–7). The function of the p53-biding site at the *PIG3* promoter was unknown until the p53-binding sequence (TGYCC)n was unraveled relatively recently (8). It is of particular interest that PIG3 shares some significant homology with oxidoreductases, a stabilizer of p53 (9), from several species, but the *PIG3* promoter acquires the full-length p53-binding sequence (TGYCC)n only in Hominoidea (8).

Abbreviations: CI, confidence interval; FPRP, false-positive reporting probability; OR, odds ratio; PIG3, p53-inducible gene 3; SCCHN, squamous cell carcinoma of the head and neck; SNP, single nucleotide polymorphism; SSCP, silver staining-based single-strand conformation.

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In response to p53, *PIG3* expression occurs much earlier, before there is a measurable signal of oxidative stress and apoptosis in the cell (5), suggesting that PIG3 is a precursor of the apoptosis pathway that determines the fate of a cell in response to cellular stress (10–12). One study showed that PIG3 contributed to early cellular response to DNA damage (7), because *PIG3* was identified in an analysis of genes induced by p53 before the onset of apoptosis (13).

It has been shown that it is the pentanucleotide microsatellite sequence (TGYCC)n (Y = C or T) within the *PIG3* promoter that interacts with p53 in activation of the PIG3-mediated apoptotic pathway (8). There are four different alleles corresponding to the numbers of pentanucleotide repeats [i.e. $(TGYCC)_{10}$, $(TGYCC)_{15}$, $(TGYCC)_{16}$ or $(TGYCC)_{17}$], and an increasing number of (TGYCC) has been shown to be correlated with increasing transcriptional activation of *PIG3* by p53 (8) but not by p53 isoforms (14) nor mutant p53 (15), which is thought to lead to an enhanced apoptotic response. This binding specificity (16) suggests that the variant alleles may play a role in cancer susceptibility (8). These findings lead us to investigate the role of this *PIG3* variable-number tandem repeats polymorphism in the etiology of head and neck cancer, an environmentally induced cancer.

Materials and methods

Study population

Details of the recruitment of squamous cell carcinoma of the head and neck (SCCHN) cases and controls have been reported elsewhere (17). Briefly, patients with histopathologically confirmed SCCHN (untreated primary tumors of the oral cavity, oropharynx, hypopharynx and larynx) were recruited in the clinic of Department of Head and Neck Surgery of The University of Texas MD Anderson Cancer Center between May 1995 and October 2002, the participation rate of eligible incident case was about 95% of those who were initially contacted for participation. Patients with primary tumors of the nasopharynx or sinonasal tract, primary tumors outside the upper aerodigstive tract, cervical metastases of unknown primary origin or histopathological diagnoses of cancer other than squamous cell carcinoma were excluded. Because few patients were of other ethnic groups, only non-Hispanic white patients were included in the case-control analysis. During the same time period, non-Hispanic white cancer-free controls were recruited from hospital visitors who were unrelated and frequency matched to the cases for age (±5 years), sex and smoking status (never, former or current), with a response rate of approximately 80% (17). After a written informed consent was obtained, blood samples were drawn from each subject after completion of personal interviews, and DNA was extracted and stored. The research protocol was approved by the institutional review boards of The University of Texas MD Anderson Cancer Center.

DNA extraction, PCR amplification, SSCP and DNA fragment analysis for genotyping and sequencing validation

From each blood sample, leukocyte cell pellet was obtained from the buffy coat layer by centrifugation of 1 ml of whole blood. The genomic DNA was extracted by using Qiagen DNA blood mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The DNA purity was detected with electrophoresis on a 1% agarose gel and the concentration was determined by UV spectrophotometry. A PCR–silver staining-based single-strand conformation (PCR–SSCP) assay was used for genotyping for all subjects. To genotype the *PIG3* variants, PCR was first used to amplify a DNA fragment located within 383–628 nt (Genbank accession number: AF010309) with the forward primer 5′-GGGCGCTGCGGTGCCAGCCTGAG-3′ and the reverse primer 5′-ACCTTCAGGAGGACTTCACC-3′. A 25 µl of PCR reaction mixture contains 50 ng genomic DNA, 6.25 pmol each primer, 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 9.0 at 25ºC and 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM each deoxyadenosine triphosphate, deoxythymidine triphosphate and deoxyguanosine triphosphate, 0.1 mM deoxycytidine triphosphate and 1 U *Taq* polymerase (Sigma, St Louis, MO). The PCR was performed by incubating the mixtures at 95ºC for 5 min, subjecting them to 35 cycles of 95ºC for 30 s, 67ºC for 45 s and 72ºC for 1 min with a final elongation step at 72ºC for 10 min. The SSCP analysis was used to analyze the PCR products. After mixing with the loading buffer (95% formamide, 20 mM ethylenediaminetetraacetic acid,

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0.05% xylene cyanol and 0.05% bromphenol blue), the PCR products were denatured by heating at 95ºC for 5 min and quenched in ice for 5 min. A 6% non-denaturing mutation detection enhancement gel (FMC BioProducts, Rockland, ME) was used for electrophoresis. The gel was dried and stained with the silver staining method. The sizes of PCR products corresponded to *PIG3* alleles 10, 15, 16 or 17 repeats are 221 bp (10 repeats), 246 bp (15 repeats), 251 bp (16 repeats) and 256 bp (17 repeats), respectively. Figure 1a shows the representative genotype pattern of *PIG3* detected by the PCR–SSCP analysis, in which 10 SSCP patterns (10/10, 10/15, 15/15, 15/16, 10/16, 10/17, 15/17, 16/16, 16/17 and 17/17) were found and further confirmed by direct sequencing. To further confirm the SSCP results, the genotypes of *PIG3* were also detected by the DNA fragment analysis for 100 SCCHN cases and 100 controls with the Genetic Analyzer 3730, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The sequences of the primers were as follows: 5'-FAM-GGGCGCTGCGGTGCCAGCCTGAG-3' (forward) and 5'-ACCTTCAGGAGGACTTCACC-3' (reverse). The PCR profile included an initial melting step of 95ºC for 5 min, 35 cycles of 95ºC for 30 s, 68ºC for 45 s and 72ºC for 1 min and a final extension step of 72ºC for 10 min. The assay success rate was >99% and the repeated sample's results were 100% concordant. Supplementary Figure S1, available at *Carcinogenesis* Online, shows the representative genotype pattern of *PIG3* repeats detected by the DNA fragment analysis, in which 10 SSCP patterns (10/10, 10/15, 15/15, 15/16, 10/16, 10/17, 15/17, 16/16, 16/17 and 17/17) were presented.

Reporter constructs, transfection and luciferase assays

The representative bands for 10, 15, 16 or 17 repeats generated by the SSCP analysis were purified with the QIAEX II gel extraction kit (Qiagen, Chatsworth, CA) according to the manufacturer's instruction. The re-amplified PCR products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). We constructed transient expression vectors containing the four different endogenous alleles of the (TGYCC)n motif in the minimum *PIG3* promoter within the microsatellite sequence (−422 to +557) (XM_039704) (Figure 2a and b). The plasmids containing the PCR fragments were sequenced to validate the sequences and pentanucleotide repeats. Two cell lines either in the presence (HCT116 *p53*+/+) or absence (HCT116 *p53*−/−) of *p53* were generously provided by Dr Bert Vogelstein (Johns Hopkins University). The cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum and antibiotics. Cell cultures were incubated at 37ºC in a humidified incubator containing 5% CO_2. For transfection, the cell lines were seeded into 24-well plates at 0.5×10^5 cells per well (BD Biosciences, Bedford, MA), and 24 h after plating, the cells were co-transfected with the FuGENE HD reagent (Roche Applied Science, Indianapolis, IN). The cells were washed and lysed with 100 µl passive lysis buffer (Promega, Madison, WI) 48 h after transfection. Results are shown as the luciferase activity relative to that of the vector control without the reporter. Both firefly and renilla luciferase activities were quantified by a Dual Luciferase Reporter Assay System (Promega), and the relative luciferase activity was calculated according to the manufacturer's instructions (BD Monolight™ 3010 Luminometer; Becton Dickinson, Mississauga, Ontario, Canada). Each co-transfection reaction contained 200 ng of pLG3-PIG3 plasmids plus 50 ng pRL-TK (Promega) plasmids that served as a transfection internal control, as shown in Figure 2C. Three replicates were analyzed for each group, the experiment was repeated at least three times and the data were presented in average with an error bar. *P* values were determined by two-sided Student's *t*-test.

Statistical analysis

Univariate analysis was performed to compare demographic profiles of the cases and controls. Subjects who had smoked >100 cigarettes in their lifetime were defined as ever smokers. The former smokers were defined as those who had reported quitting smoking for at least 1 year prior to interview (for the controls) or diagnosis (for the cases). Subjects who had reported drinking alcoholic beverages at least once a week and longer than 1 year prior to diagnosis or interview were defined as ever drinkers. Those who had quit drinking for longer than 1 year prior to diagnosis or interview were defined as former drinkers. The odds ratios (ORs) and their 95% confidence intervals (CIs) for the *PIG3* genotype were calculated by logistic regression analysis with and without adjustment for age (in years), sex, smoking status and alcohol use. For logistic regression analysis, the *PIG3* genotype was recoded as a dummy variable, and for a tend test, the *PIG3* genotype was recoded according to a dominant model assuming an allele dosage of the variant. All statistical analysis was performed with Statistical Analysis System software (Version 8; SAS Institute, Cary, NC).

Results

Frequency distribution of the PIG3 (TGYCC)n motif and alleles in four ethnic groups

We used a non-radioactive, PCR-based genotyping assay with silver staining to visualize the variants of the *PIG3* promoter (TGYCC)n motif (Figure 1a), followed by sequencing (Figure 1b) of the bands of different genotype patterns identified on the gel for each representative sample. These samples of different genotypes were selected from genotyping data on the variants in the *PIG3* promoter (TGYCC)n motif in the initial 400 subjects, including 100 non-Hispanic whites, 100 African Americans, 100 Hispanic Americans and 100 Native Chinese frequency matched on age and sex. Each group included 80 males and 20 females

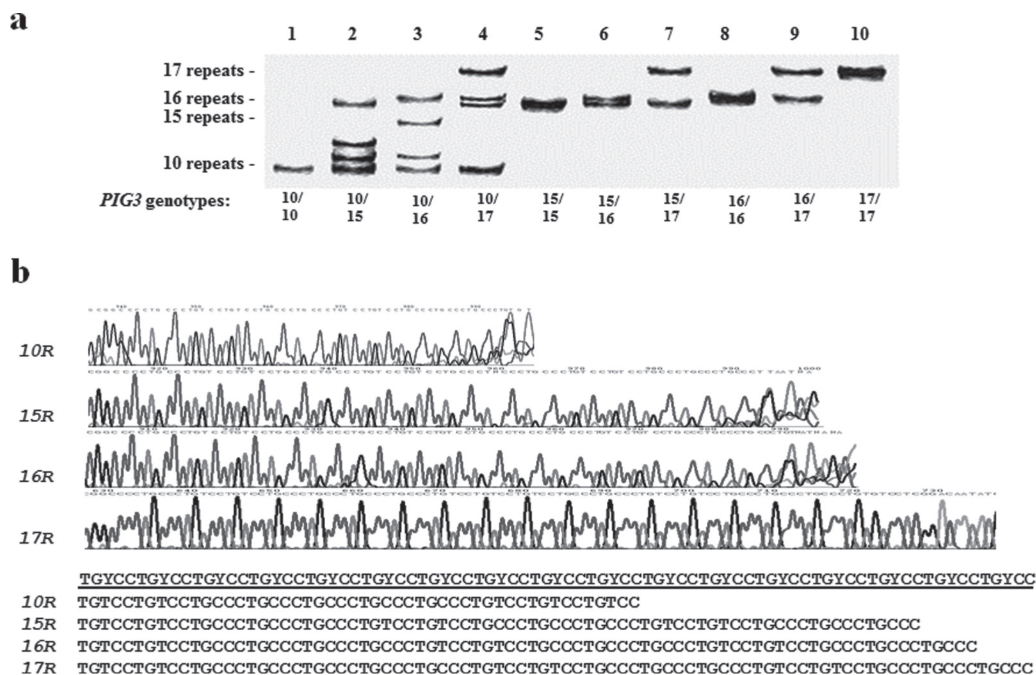

Fig. 1. *PIG3* polymorphism defined by the promoter (TGYCC)n motif in the population. *PIG3* (TGYCC)n genotypes detected by PCR–SSCP analysis (**a**), sequence confirmation of PIG3 (TGYCC)n alleles identified in (**b**).

frequency matched on age in years, mean age (standard deviation): 57.59 (12.35), 57.55 (7.28), 57.54 (9.81) and 57.55 (8.60), respectively. Based on the sequence data, we confirmed the four previously described *PIG3* alleles [i.e. $(TGYCC)_{10}$, $(TGYCC)_{15}$, $(TGYCC)_{16}$ and $(TGYCC)_{17}$] and 10 different genotypes (Figure 1b). As shown in Table I, we found that the 15 repeats was the most common allele and the 16 repeats was secondary major common allele, whereas the 10 and 17 repeats were two rare alleles. The $(TGYCC)_{15}$, which was considered the wild-type allele (Table I), was common in non-Hispanic whites (0.66), African Americans (0.72), Hispanic Americans (0.72) and Native Chinese (0.78), and the difference between non-Hispanic whites (the lowest) and Native Chinese (the highest) was statistically significant ($P = 0.016$). Consistent with our findings, the published data also showed that the $(TGYCC)_{15}$ allele was frequent among Greek (0.59), British (0.58) (18) and German (0.62) (8) populations and more common in Japanese populations (0.89) (19). However, $(TGYCC)_{12}$, $(TGYCC)_{14}$ and $(TGYCC)_{18}$ were not observed in this study, nor were present in other populations (8,18), as were reported in Japanese populations (19).

Association between PIG3 promoter polymorphic repeats and risk of SCCHN

To evaluate the association between the *PIG3* promoter (TGYCC)n motif and SCCHN risk, we genotyped for the variants in the *PIG3* promoter (TGYCC)n motif in 616 patients with SCCHN and 623 cancer-free controls. All these subjects were self-reported non-Hispanic whites and were predominantly males (76.1% of the cases and 72.6% of the controls; $P = 0.149$). The mean age was 56.4 years (±standard deviation, 11.8 with a range of 20–90 years) for the cases and 56.6 years (±12.1 with a range of 19–87 years) for the controls, and the difference was not statistically significant ($P = 0.151$). However, there were more current smokers (35.1%) or current drinkers (51.6%) in the cases than in the controls (26.0% and 42.0%, respectively) and the differences were statistically significant ($P < 0.001$) (Table II). Therefore, they were further adjusted for in the multivariate logistic regression analysis. Histological subtypes and tumor sites of the cases included 195 squamous cell carcinomas of the oral cavity (31.7%), 301 of the pharynx (48.8%, including 265 oropharynx and 36 hypopharynx) and 120 of the larynx (19.5%).

As shown in Table III, the frequencies of four *PIG3* promoter (TGYCC)n alleles with 10, 15, 16 and 17 repeats in the 623 controls were 3.4%, 64.5%, 18.6% and 13.5%, respectively, which differed from that in the cases (3.7%, 57.9%, 23.9% and 14.5%, respectively). As expected, only the frequency of the *PIG3* promoter $(TGYCC)_{15}$ (wild-type) allele between the cases (57.9%) and controls (64.5%) was statistically significantly different, and further adjustment for age, sex, smoking and alcohol use did not change the estimates substantially (Table III). When the *PIG3* promoter $(TGYCC)_{15}$ homozygotes were used as the reference group, those who did not carry the *PIG3* promoter $(TGYCC)_{15}$ allele had a significantly increased risk of cancer [OR = 1.34; 95% CI = 1.04–1.73 for heterozygotes and OR = 1.69; 95% CI = 1.18–2.44 for non-$(TGYCC)_{15}$ carriers], and the trend testing showed that risk increased as the number of $(TGYCC)_{15}$ alleles decreased ($P_{Trend} = 0.002$).

Table I. Frequency distribution of the *PIG3* (TGYCC)n motif and alleles in four ethnic groups of non-Hispanic whites, African Americans, Hispanic Americans and Native Chinese[a]

PIG3 (TGYCC)n motif	Non-Hispanic whites		African Americans		Hispanic Americans		Native Chinese	
	No.	%	No.	%	No.	%	No.	%
No. of subjects	100		100		100		100	
Allele frequency	200		200		200		200	
10	10	0.050	5	0.025	6	0.030	7	0.035
15	132	0.660	144	0.720	144	0.720	157	0.785
16	34	0.170	34	0.170	39	0.195	27	0.135
17	24	0.120	17	0.085	11	0.055	9	0.045
P^b	Reference		0.336		0.082		0.016	

[a]Each group included 80 males and 20 females frequency matched on age in years. The mean age (standard deviation) for Non-Hispanic whites, African Americans, Hispanic Americans and Native Chinese: 57.59 (12.35), 57.55 (7.28), 57.54 (9.81) and 57.55 (8.60), respectively.
[b]Two-sided χ^2 test.

Table II. Distribution of selected variables in SCCHN patients and controls in non-Hispanic whites

Variable	Cases (*N* = 616)		Controls (*N* = 623)		*P* value[a]
	No.	%	No.	%	
Age (years)					
<50	170	27.6	176	28.2	0.151
50–60	201	32.6	173	27.8	
>60	245	39.8	274	44.0	
Sex					
Male	469	76.1	452	72.6	0.149
Female	147	23.9	171	27.5	
Tobacco smoking					
Never	156	25.3	202	32.6	<0.001
Former	244	39.6	258	41.4	
Current	216	35.1	162	26.0	
Alcohol drinking					
Never	123	20.0	198	31.8	<0.001
Former	174	28.4	163	26.2	
Current	318	51.6	262	42.0	
Tumor sites					
Oral cavity	195	31.7			
Oropharynx	265	43.0			
Hypopharynx	36	5.8			
Larynx	120	19.5			

[a]Two-sided χ^2 test.

Table III. Association between the *PIG3* polymorphic (TGYCC)n motif and risk of HNSCC

	Cases		Controls		Adjusted OR[a] (95% CI)
	No.	%	No.	%	
PIG3 allele	*N* = 1232		*N* = 1246		
$(TGYCC)_{10}$	45	3.7	42	3.4	
$(TGYCC)_{15}$	713	57.9	804	64.5	
$(TGYCC)_{16}$	295	23.9	232	18.6	
$(TGYCC)_{17}$	179	14.5	168	13.5	
χ^2 test[b]	$P = 0.004$				
PIG3 genotype[c]	*N* = 616		*N* = 623		
Homozygote $(TGYCC)_{15}$	199	32.3	258	41.4	1.00
Heterozygote	315	51.1	288	46.2	1.34 (1.04–1.73)
Non-$(TGYCC)_{15}$	102	16.6	77	12.4	1.69 (1.18–2.44)
χ^2 test[d]	$P = 0.002$				$P_{Trend} = 0.002$

[a]ORs and 95% CI for the genotype with adjustment for age, sex, smoking and alcohol use.
[b]Test for the distribution of different (TGYCC)n alleles with adjustment for age, sex, smoking and alcohol use.
[c]The genotype distribution of the controls is consistent with the expected one from the Hardy-Weinberg Equilibrium model based χ^2 test with 1 degree of freedom.
[d]Trend test for the genotypes with different variant alleles (0, 1 and >1) with adjustment for age, sex, smoking and alcohol use.

Variation in p53-modulated transcriptional activity of PIG3 promoter with various (TGYCC)n motifs

Because we found that individuals who carried genotypes containing the *PIG3* motif other than $(TGYCC)_{15}$ had an increased SCCHN

risk, compared with the *PIG3* promoter $(TGYCC)_{15}$ homozygotes, we further used the luciferase assays to test the hypothesis that $(TGYCC)_{15}$ homozygous promoter has the highest transcriptional activity than all other alleles. To test this hypothesis, we constructed transient expression vectors containing the four different endogenous alleles of the (TGYCC)n motif in the minimum *PIG3* promoter within the microsatellite sequence (−422 to +557) (XM_039704) (Figure 2a and b), with which we investigated the role of the (TGYCC)n motif in modifying the p53-mediated *PIG3* promoter activity by the luciferase reporter gene assay. As shown in Figure 2c, the vector having the *PIG3* $(TGYCC)_{15}$ motif had the highest level of expression or promoter activity as measured by the luciferase assay either in the presence (i.e. in HCT116 *p53*+/+ cells) or absence (i.e. in HCT116 *p53*−/− cells) of p53, compared with the other three (TGYCC)n motifs, although the expression was clearly p53 dependent in both cell lines. However, we did not observe a direct linear correlation between the expression levels and the number of the (TGYCC)n motif in this experimental system, a finding appears to be not consistent with the previous report (8).

The data derived from the colon cancer HCT116 cells represented the expression status of the *PIG3* promoter with different number of (TGYCC)n motif in the presence or absence of p53 without any cellular stress. However, in the previous report with the biliary cancer CAK-1 cells (8), the *PIG3* promoter activity appeared to be increased as the number of the *PIG3* (TGYCC)n motif increased in the presence of experimentally induced p53 overexpression, a scenario that may mimic cellular stress response. The latter finding may be more relevant to environmental exposure to carcinogens in the etiology of cancer, although it is also probable that cell-type specificity may contribute to the discrepancy observed between our colon cancer cells and biliary cancer CAK-1 cells.

We further calculated the false-positive reporting probability (FPRP) values at different prior probability levels for all significant association findings (Table IV). For a prior probability of 0.10, assuming the OR for a specific genotype is 1.5, the FPRP value was 0.182 for an association between heterozygous genotypes and risk of SCCHN with a statistical power of 0.999, and the FPRP value for an association with SCCHN risk and non-(TGYCC)15 was 0.053 with a statistical power of 0.816, which is a worthy finding.

Finally, we summarized the allele frequencies of both cases and controls in several populations in previously published studies in Supplementary Table SI, available at *Carcinogenesis* Online. It is clear from the summarized data that the *PIG3* $(TGYCC)_{15}$ is the most frequent allele in different populations and the repeats other than *PIG3* $(TGYCC)_{15}$ should be considered its variants. The observed frequency (64%) in our 1246 chromosomes of white controls was close to that from two other white control populations (57.8% from 346 chromosomes and 57.6% from 370 chromosomes), although this frequency was higher in a Japanese population (88.6 from 676 chromosomes), which is close to that (78% from 200 chromosomes) of a Chinese population we found (Table I).

Discussion

p53 acts as a tumor suppressor through several different mechanisms, including cleaning the susceptible cells through sensing the extent of DNA damage, regulating cell cycle for DNA repair and participating in reactive oxygen species-apoptosis mechanism to activate the apoptotic pathway (11,12). PIG3 is a newly identified p53-dependent molecule that is also involved in the process of apoptosis (7,20). It is known that PIG3 induces apoptosis in response to the accumulation of reactive oxygen species, and the increasing number of the repeats

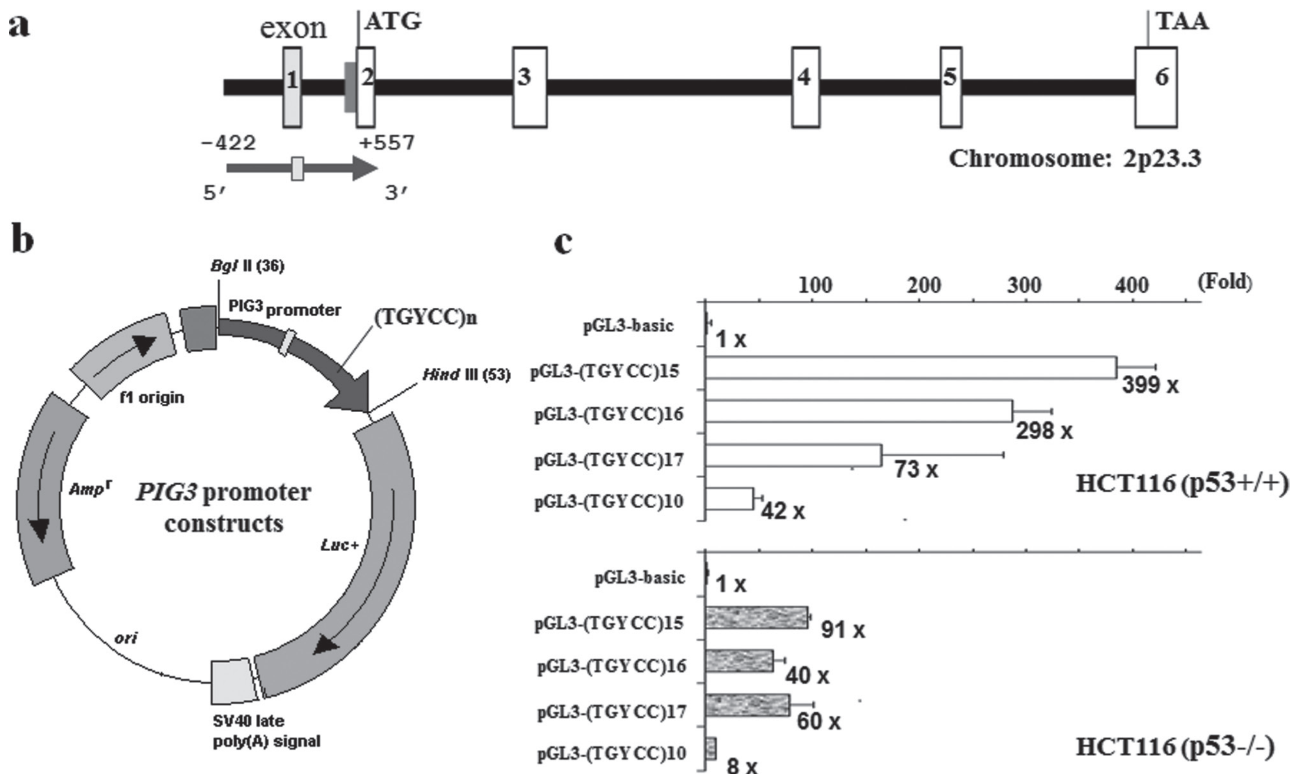

Fig. 2. Constructs containing the *PIG3* (TGYCC)n alleles and results of the luciferase assay. Genomic structure of the *PIG3* gene and location of (TGYCC)n motif (**a**), the reporter constructs generated by directional cloning (**b**), differential transcriptional activation by the constructed *PIG3* promoter. Expression of the reporter constructs that contain the minimal *PIG3* promoter with different (TGYCC)n motif were transfected into the HCT116 cells (p53−/− or p53+/+), then measured by the luciferase assay (**c**). Note: (a) The gray horizontal bar represents gene length and white (or yellow) boxes represent exons with start codon ATG and stop codon TAA; (b) A transient expression vector with an insert location for (TGYCC)n in the *PIG3* promoter; (c) Three replicates were analyzed for each group, the experiment was repeated at least three times, and the data were presented in average with an error bar.

Table IV. FPRP value for associations between *PIG3* (TGYCC)n motif and HNSCC risk

Risk	OR (95%CI)[a]	*P* value	Statistical power[b]	Prior probability			
				0.2500	0.1000	0.010	0.001
$(TGYCC)_{15}$ heterozygote	1.34 (1.04–1.73)	0.002	0.999	0.069	0.182	0.710	0.961
Non-$(TGYCC)_{15}$	1.69 (1.18–2.44)	0.002	0.816	0.018	0.053	0.383	0.862

[a]The crude OR reported in Table III.
[b]Calculated using study subjects to detect an OR of 1.5.

(TGYCC)n of a polymorphic microsatellite in the *PIG3* promoter, a known p53-binding site, increases transcriptional activation of *PIG3* by p53, leading to an enhanced apoptotic response (5). Therefore, it is suggested that inheritance of this microsatellite may affect the individual's susceptibility to cancer (8).

In this study, we have demonstrated that the $(TGYCC)_{15}$ (the most common wild-type allele) within the *PIG3* promoter is associated with a decreased risk of SCCHN. This finding was further supported by additional experiments that showed that $(TGYCC)_{15}$ was associated with the highest levels of expression under control of the *PIG3* gene promoter. However, our findings do not support previous findings from some smaller studies. For example, no significant association was found between (TGYCC)n repeats and risk of breast cancer (167 cases and 330 controls) and lung cancer (231 cases and 515 controls) in both Greek and British populations (18), in which the $(TGYCC)_{15}$ homozygotes were also used as the reference. Another study did not find an association with bladder cancer in a Japanese population (273 cases and 338 controls) (19), in which a short repeat subtype that harbored at least one allele of 14 or less repeats, not the $(TGYCC)_{15}$ homozygotes, were used as the reference. In addition to smaller study sizes included in these studies, another possible explanation is that there may be ethnic and geographic differences in the etiology of different cancers.

We further investigated the underlying molecular mechanism of $(TGYCC)_{15}$ for decreased SCCHN risk. Functional analysis based on luciferase reporter gene experiments indicated that $(TGYCC)_{15}$, the most common allele, led to the most effective transcriptional activity of the promoter, whereas the other three variant alleles were associated with lower transcriptional activities and thus increased risk of cancer. The functional data suggest that individuals carrying the genotypes containing the $(TGYCC)_{15}$ allele should have a better apoptotic potential in response to stress signals, which is consistent with a reduced risk of cancer, as we observed consistently in this study. More importantly, our epidemiological data were consistent with our experimental data generated from the HCT116 cells, which support the notion that among these three genotypes, the '15/15' genotype is predicted to be associated with the highest promoter transcriptional activity, the highest level of apoptotic potential and thus the lowest risk of SCCHN.

However, this prediction based on the results from our *in vitro* assays using colon cancer cells is somewhat different from the prediction based on the data generated from the CAK-1 cells (derived from a human renal cell carcinoma) in which greater number of the *PIG3* (TGYCC)n motif was predicted to have a greater apoptotic response (8). This discrepancy could be due to cell-specific events or the differences in the p53-*PIG3* promoter binding in different experimental systems. However, this study did provide further support for a link between functional phenotype associated with genetic variation in *PIG3* and cancer risk phenotype as predicted by putative functions of the polymorphic *PIG3* (TGYCC)n motif.

Finally, the associations between (TGYCC)n and cancer risk were assessed in previously reported case-control studies using a different reference group, leading to inconsistent results of risk estimates (18,19). However, our large sample size for risk assessment and additional laboratory experiments generated consistent findings, that is, the observed increase in cancer risk is associated with rare variant genotypes that may be associated with low expression of *PIG3*, which has been shown to be associated with a low level of apoptosis (5), leading to a high probability of carcinogenesis.

To our knowledge, this is the first study to evaluate the associations between polymorphic *PIG3* promoter (TGYCC)n motif and risk of SCCHN. However, this study also has several limitations. First, this hospital-based case-control study included controls who were not randomly selected from the same population as the cases come from. Second, HPV16, one of the major risk factors for SCCHN, was not evaluated for patients included in this study. The joint effects of HPV16 and *PIG3* genotypes should be explored by additional prospective studies with well-designed clinical investigations.

In summary, this study provides evidence that the variant genotypes of (TGYCC)n repeats in the *PIG3* promoter are functional and associated with risk of SCCHN in a non-Hispanic white population. However, large studies are needed to validate our findings.

Supplementary material

Supplementary Table S1 and Figure S1 can be found at http://carcin.oxfordjournals.org/

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