Carcinogenesis vol.34 no.7 pp.1551–1557, 2013
doi:10.1093/carcin/bgt096
Advance Access publication March 18, 2013

Genetic variants in *p53*-related genes confer susceptibility to second primary malignancy in patients with index squamous cell carcinoma of head and neck

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Because of their important roles in mediating the stabilization and expression of p53, we hypothesized that high-risk genotypes of polymorphisms in *p53*-related genes, including *p53*, *p73*, *p14^{ARF}*, *MDM2* and *MDM4*, may be associated with an increased risk of second primary malignancy (SPM) after index squamous cell carcinoma of the head and neck (SCCHN). We analyzed data from a cohort of 1283 patients with index SCCHN who were recruited between 1995 and 2007 at MD Anderson Cancer Center and followed for SPM development. Patients were genotyped for nine polymorphisms of *p53*-related genes. A log-rank test and Cox models were used to compare SPM-free survival and risk. Our results demonstrated that each *p53*-related polymorphism had a moderate effect on increased SPM risk, but when we combined risk genotypes of these nine polymorphisms together, we found that SPM-free survival was significantly shorter among risk groups with a greater number of combined risk genotypes. SPM risk increased with increasing number of risk genotypes ($P < 0.0001$ for trend). Compared with the low-risk group (0–3 combined risk genotypes), both the medium-risk (4–5 combined risk genotypes) and high-risk (6–9 combined risk genotypes) groups had significantly increased SPM risk [hazard ratio (HR): 1.6; 95% confidence interval (CI): 1.0–2.6 and HR: 3.0; 95% CI: 1.8–5.0, respectively]. Moreover, such significant associations were even higher in several subgroups. Our findings suggest that combined risk genotypes of *p53*-related genes may jointly modify SPM risk, especially in patients who are smokers and those with index non-oropharyngeal cancers. However, larger studies are needed to validate our findings.

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is characterized by highly aggressive local–regional tumor growth and results in significant morbidity. Despite the advances in loco-regional control achieved with modern treatment, the survival of patients with SCCHN has not essentially improved, partly because about 20% of these patients develop a second primary malignancy (SPM) (1–4). Although drinking alcohol and smoking cigarettes have been linked to SPM risk, patients with SCCHN do not always develop a SPM, even if they continue to consume alcohol and cigarettes (5–7). This suggests that genetic susceptibility may also contribute to SPM etiology. Identifying genetic markers associated with SPM in patients with SCCHN would allow for the identification of a subpopulation of SCCHN survivors with a high risk of SPM.

Tobacco and alcohol consumption prior to the diagnosis of the primary SCCHN significantly increases the risk of SPM (8). Tobacco smoke can cause DNA damage that deregulates cell cycle control and apoptosis, which results in carcinoma if the DNA damage is left unrepaired (9,10), and genetically inherited DNA repair capacity can modulate individual susceptibility to tobacco-induced carcinogenesis (11,12). The *p53* molecular pathway plays a central role in maintaining genomic integrity and protecting cells against such damage (13).

p53, a tumor suppressor known as 'the guardian of the genome', plays an important role in the prevention of carcinogenesis induced by DNA damage from various agents (14). *p53*-Related genes, such as *p73*, *p14* and murine double minute proteins 2 and 4 (*MDM2* and *MDM4*), respond to a variety of stress signals to affect cellular homeostatic mechanisms (15,16). Functionally, *p73*, located on the 1p36 locus, activates transcription of *p53*-responsive genes (17,18). *p14^{ARF}*, which represents the product of exons 1b, 2 and 3 of the CDKN2A locus on 9p21, interacts directly with *MDM2*, thereby indirectly regulating the level of p53 (19). *MDM2*, described as the first p53 E3-ubiquitin ligase, induces polyubiquitylation and degradation of p53 when overexpressed (20). *MDM4* is a negative regulator of p53 and cooperates with *MDM2* to inhibit p53 activity in the cellular response to DNA damage (21).

The putatively functional polymorphisms in these *p53*-related genes (*p53*, *p73*, *p14^{ARF}*, *MDM2* and *MDM4*), which regulate the cell cycle and apoptosis, may collectively modify genetic susceptibility to primary SCCHN or SPM after index SCCHN (17–21). The *p53* codon 72 at exon 4 encodes either a proline (Pro) or arginine (Arg) that appears to influence individual susceptibility to cancer by functionally affecting the p53 protein. The non-coding exon 2 polymorphism of *p73* G>A rs2273953 may functionally affect the p73 protein by affecting the efficiency of *p73* translation initiation. Since the two *p14^{ARF}* polymorphisms are within the functional regions of the gene's promoter (*p14^{ARF}*-rs3731217) and 3′-untranslated region (UTR) (*p14^{ARF}*-rs3088440), we speculated that they may potentially affect p14^{ARF} expression levels by altering the efficiency of translational initiation. Among the polymorphisms of *MDM2*, two polymorphisms in promoter, *MDM2*-rs2279744 and *MDM2*-rs937283, may lead to change of MDM2 transcription levels, resulting in altered p53-MDM2 binding affinity and regulation of cell cycle control. Unlike p53, p73 and MDM2, few studies have investigated the role of *MDM4* variants in the risk of human cancers. We identified three common tagging SNPs, rs11801299 G>A and rs1380576 C>G in 3′-UTR and rs10900598 G>T in 5′-UTR, which may alter or influence MDM4 expression and subsequently increase susceptibility to cancer.

We have reported previously that SPM risk among SCCHN patients was associated with individual genetic variants of these *p53*-related genes. In these studies, we found that the variant genotypes of *p53* codon 72, *p14^{ARF}*-rs3731217 and *p14^{ARF}*-rs3088440 polymorphisms were associated with a significantly increased SPM risk compared with their corresponding homozygous wild-type genotypes, respectively [hazard ratio (HR): 1.58, 95% confidence interval (CI): 1.07–2.34 for *p53* codon 72; HR: 1.48, 95% CI: 1.00–2.19 for *p14^{ARF}*-rs3731217

Abbreviations: CI, confidence interval; HR, hazard ratio; LD, linkage disequilibrium; MDM, murine double minute protein; SCCHN, squamous cell carcinoma of the head and neck; SPM, second primary malignancy; UTR, untranslated region.

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and HR: 1.61, 95% CI: 1.07–2.43 for $p14^{ARF}$-rs3088440, respectively], but a significantly reduced SPM risk for *p73* G>A rs2273953 polymorphism (HR: 0.59; 95% CI: 0.39–0.89) (17–19). However, the similar associations have not been assessed for the two promoter polymorphisms of *MDM2*: *MDM2*-rs2279744 and *MDM2*-rs937283 and the three common tagging SNPs of *MDM4*: *MDM4*-rs11801299 G>A and *MDM4*-rs1380576 C>G in 3′-UTR and *MDM4*-rs10900598 G>T in 5′-UTR. Given the role of each of these variants in regulation of cell cycle and apoptosis and genetic susceptibility to primary SCCHN or SPM after index SCCHN (17–21), we hypothesize that these nine polymorphisms collectively modify the risk of SPM and that these combined risk genotypes could serve as susceptibility markers for identifying high-risk subgroups of patients who might benefit from personalized prevention and treatment, and we evaluated the combined effects of these polymorphisms on risk of SPM in a well-established cohort of SCCHN patients.

Materials and methods

Study subjects

We analyzed data from a cohort of 1283 patients with index SCCHN who were consecutively recruited between May 1995 and January 2007 as part of an ongoing prospective molecular epidemiological study at The University of Texas MD Anderson Cancer Center, as described previously (18). All subjects provided Institutional Review Board-approved informed consent and were recruited regardless of age, sex, ethnicity or cancer stage. The exclusion criteria included any previous cancer (except non-melanoma skin cancer), distant metastases at presentation, primary sinonasal cancers, salivary gland cancers, cervical metastases of unknown origin and cancers outside the upper aerodigestive tract. Patients were monitored throughout their treatment and post-treatment course with regularly scheduled clinical and radiographic examinations.

All patients were interviewed at presentation for completion of an epidemiological questionnaire that included data on alcohol and smoking status. Alcohol status was categorized as 'ever drinkers' (those who had drunk at least one alcoholic beverage/week for at least 1 year during their lifetimes) or 'never drinkers' (those who never had such a pattern of drinking). Smoking status was categorized as 'ever smokers' (those who had smoked at least 100 cigarettes in their lifetimes) or 'never smokers' (those who had smoked fewer than 100 cigarettes in their lifetimes).

Additional clinical data were obtained from review of the patients' medical records at initial presentation and follow-up, including overall stage, site and treatment at presentation of the index tumor. A SPM was carefully defined according to the modified criteria of Warren *et al.* (22). Briefly, SPMs were considered if the second lesions had different histopathologic types or if they developed over 5 years after treatment for the index tumor and/or clearly separated by normal epithelium according to clinical and radiographic assessment. Pulmonary lesions were included as a SPM if they had a non-squamous histology or if they were isolated squamous lesions over 5 years from index SCCHN and considered by both thoracic oncologist and thoracic surgeon as a SPM. If there was discrepancy or difference in opinions regarding recurrence or SPM, the second lesion was not considered a SPM but a local recurrence. SPMs were categorized as tobacco-associated (SCCHN or cancers of the esophagus, lung or bladder) or non-tobacco-associated SPM (prostate cancer, papillary thyroid carcinoma, colon adenocarcinoma, etc.).

Genotyping

Genomic DNA was extracted from patient blood samples and genotyped for the following polymorphisms: *p53* codon 72, *p73* G4C14-to-A4T14, $p14^{ARF}$-rs3731217, $p14^{ARF}$-rs3088440, *MDM2*-rs2279744, *MDM2*-rs937283, *MDM4*-rs11801299, *MDM4*-rs1380576 and *MDM4*-rs10900598. The details of genotyping for these polymorphisms have been described previously (17–21). There was 100% concordance when 10% of the genotyping assays were repeated.

Statistical analysis

The primary endpoint of the study was SPM occurrence. Time-to-event was calculated from the date of diagnosis of the index SCCHN to the date of diagnosis of the SPM. Student's *t*-test was used to compare the mean age and follow-up time between the patients who developed an SPM and those who did not. The chi-square test was used to assess the differences in ethnicity, sex, smoking and alcohol status, primary tumor site and stage, treatment, and genotype distributions between the two groups. Kaplan–Meier curves were used to estimate SPM-free survival, and the log-rank test was used to evaluate significant differences ($\alpha = 0.05$) in SPM-free survival between the different genotyping groups. In light of the crossed over Kaplan–Meier curves, we also applied alternative tests to the log-rank test for testing homogeneity of the survival functions. Specifically, we applied the Wilcoxon (Gehan's) test to investigate the survival difference at the early stage of follow-up, whereas we used Fleming-Harrington's test (two parameters $p = q = 0.5$) to investigate the survival difference at the late stage of follow-up (23). In the univariate logistic regression analysis, we estimated the association between risk of SPM and selected demographic variables, risk factors and clinical variables by computing the HRs and their 95% CIs. The associations between individual epidemiological factors, clinical characteristics, and treatment variables, and time-to-event (SPM), were initially assessed using univariate Cox proportional hazards regression models. The data were consistent with the assumptions of the Cox proportional hazards regression model from the examination of Kaplan–Meier survival curves and log-minus-log survival plots (24,25). In the multivariable logistic regression models, adjusted for age, sex, ethnicity, smoking and alcohol consumption, we evaluated the effects of single polymorphism or combined polymorphisms of *p53*-related genes on the risk of SPM. The joint effects were further stratified by smoking status, index tumor site and SPM site. Statistical analyses were performed using Statistical Analysis System (SAS) software (version 9.1.3; SAS Institute, Cary, NC). For all analyses, statistical significance was set at $P < 0.05$, and all tests were two sided.

Results

Patient characteristics

Demographic, risk and clinical characteristics of the 1283 patients (overall and according to SPM occurrence) are shown in Table I. With a median follow-up time of 34.0 months (range, 2.4–142.4 months), 1163 patients remained SPM free, whereas 120 patients developed SPMs. Of these 120 patients, 85 developed SPMs at tobacco-associated sites and 35 patients developed SPMs at other sites. Although this patient cohort included predominantly men (76.0%), sex was not associated with SPM development ($P = 0.5285$). We did not observe significant differences between patients with and without SPMs with regard to smoking history ($P = 0.1204$), alcohol consumption ($P = 0.3442$), index cancer site ($P = 0.3184$), index cancer stage ($P = 0.6926$) or treatment ($P = 0.8832$). However, compared with SPM-free patients, patients who developed SPMs were more likely to be older ($P < 0.0001$) and non-Hispanic whites ($P = 0.0440$).

Combined effects of the p53-related genetic variants on risk of SPM

Because each of these polymorphisms appeared to have a minor or moderate effect on SPM risk (Table II) and no polymorphisms were in linkage disequilibrium (LD) to each other between variants belonging to the same genes (data not shown), we categorized the nine polymorphisms under investigation into a new variable. Specifically, in the study subjects who had data available on all nine polymorphisms, we categorized all putative risk (HRs > 1.0) genotypes of each polymorphism into a new variable according to the number of risk genotypes carried by an individual for each of the nine polymorphisms in a dominant model (for the *p73* G>A rs2273953 and *MDM4*-rs10900598 G>T genotypes, we reversed the reference group to reflect the protective effects of the variant genotypes: *p73* GA/AA and *MDM4*-rs10900598 GT/TT). Therefore, according to the number of risk genotypes carried by each individual and the level of SPM risk linked to the risk genotypes of each individual polymorphism, we categorized the individuals into different risk groups with different combined risk genotypes to evaluate the collective effects of the *p53*, *p73*, *p14*, *MDM2* and *MDM4* polymorphisms on the risk of SPM as shown in Table III. When we combined the risk genotypes of the nine polymorphisms together, we found that SPM-free survival decreased significantly as the number of combined risk genotypes increased (log-rank test; $P < 0.0001$) (Figure 1). In light of the crossed over Kaplan–Meier curves, we used other alternative tests, such as Wilcoxon (Gehan's) and Fleming-Harrington's test, to the log-rank test; and all were highly significant (all *P*-values < 0.0001), indicating that there was survival difference between different groups. As shown in Table III, there was a significant trend in SPM risk ($P < 0.0001$ for trend) between increased SPM risk and the increasing number of

Table I. Distribution of selected participant characteristics

Characteristic	Total		SPM free		SPM		*P*-value[a]
	N	%	*n*	%	*n*	%	
Total patients	1283	100	1163	90.7	120	9.3	
Age							
<Median (57 years)	662	51.6	623	53.6	39	32.5	<0.0001
>Median (57 years)	621	48.4	540	46.4	81	67.5	
Sex							
Male	975	76.0	881	75.7	94	78.3	0.5285
Female	308	24.0	282	24.3	26	21.7	
Ethnicity							
Non-Hispanic white	1086	84.6	992	85.3	94	78.3	0.0440
Other	197	15.4	171	14.7	26	21.7	
Smoking							
Never	344	26.8	319	27.4	25	20.8	0.1204
Ever	939	73.2	844	72.6	95	79.2	
Alcohol							
Never	335	26.1	308	26.5	27	22.5	0.3442
Ever	948	73.9	855	73.5	93	77.5	
Index cancer site							
Oral cavity	416	32.4	378	32.5	38	31.7	0.3184
Oropharynx	572	44.6	524	45.0	48	40.0	
Larynx/hypopharynx	295	23.0	261	22.5	34	28.3	
Index cancer stage							
1 or 2	323	25.2	291	25.0	32	26.7	0.6926
3 or 4	960	74.8	872	75.0	88	73.3	
Treatment							
Surgery only	229	17.8	208	17.9	21	17.5	0.8832
Surgery plus adjuvant treatment	318	24.8	285	24.5	33	27.5	
XRT[b]	328	25.6	300	25.8	28	23.3	
XRT plus chemotherapy	408	31.8	370	31.8	38	31.7	

[a]*P*-values were calculated from chi-square tests.
[b]XRT: radiotherapy.

combined risk genotypes; in particular, patients with eight or nine risk genotypes had an ~7-fold (HR: 7.1; 95% CI: 2.4–21.0) higher risk of SPM compared with patients carrying 0–3 risk genotypes.

We further categorized the patients into three main groups: (i) the low-risk group (0–3 combined risk genotypes); (ii) the medium-risk group (4–5 combined risk genotypes) and (iii) the high-risk group (6–9 combined risk genotypes). The SPM-free survival differed significantly among the three risk groups (log-rank test; $P < 0.0001$) (Figure 2). Furthermore, the patients in the medium-risk and high-risk groups had a 1.6-fold (HR: 1.6; 95% CI: 1.0–2.6) and 3.0-fold (HR: 3.0; 95% CI: 1.8–5.0) increased risk of SPM compared with those in the low-risk group (Table III). When the similar analysis was performed among non-Hispanic whites only, we found that the results were similar when SPM risk was limited to non-Hispanic whites (data not shown).

Stratification analysis of the combined p53-related genetic variants with risk of SPM

We further evaluated the associations between the combined risk genotypes and risk of SPM stratified by age, smoking status, index cancer site and SPM type, as summarized in Table IV. When we used the low-risk group as comparison group, a significant higher HR was observed among young patients (HR: 4.2; 95% CI: 1.7–10.4) than among older patients (HR: 2.3; 95% CI: 1.3–4.4) in the high-risk group, whereas the interaction between age and combined risk genotypes on risk of SPM was not significant ($P_{int.} = 0.100$ for the six risk groups and $P_{int.} = 0.190$ for the three risk groups). Compared with the low-risk group, the increased risk of SPM was statistically significant for ever smokers in both the medium-risk group (HR: 1.7; 95% CI: 1.0–2.9) and high-risk group (HR: 3.0; 95% CI: 1.7–5.4). The increase in risk was also higher for patients who had index non-oropharynx tumors in both the medium-risk (HR: 2.4; 95% CI: 1.2–4.8) and high-risk (HR: 5.0; 95% CI: 2.4–10.5) groups as well as for SPMs at either tobacco-associated sites or non-tobacco-associated sites (HR: 3.0; 95% CI: 1.6–5.5 for SPM at tobacco-associated sites and HR: 3.3; 95% CI: 1.3–8.3 for SPM at non-tobacco-associated sites).

Discussion

It is well established that genes in the *p53* pathway play a critical role in DNA repair, apoptosis and cell cycle regulation to conserve genomic stability and prevent mutations induced by tobacco and other carcinogens (13,15). Single nucleotide polymorphisms of these genes may affect their corresponding protein expression or function and, thus, potentially affect cancer risk or risk of SPMs (17–21,26,27). In the present study, we found a significant association between the combined risk genotypes of *p53*-related genes and the risk of developing SPM following index SCCHN.

Interactions among these genes in the *p53* pathway are involved in the regulation of *p53* activity, which likely provides biological plausibility for the observed associations between these polymorphisms and SPM risk. For example, the polymorphism *p73* G>A rs2273953 is completely linked with another nearby variant, *p73* C>T rs1801173, which form a stem-loop structure and may influence p73 expression. It was reported that the enhanced binding of p53 codon 72 to *p73* can neutralize *p73*-induced apoptosis (28), suggesting a possible interaction between *p73* and *p53* polymorphisms in the development of human cancers. Our group also reported that an increased risk of SPM after index SCCHN was associated with *p53* and *p73* polymorphisms both individually and in combination (29). $p14^{ARF}$, which plays an important role in the *ARF-MDM2-p53* pathway, releases *p53* by binding to and inactivating the MDM2 protein, resulting in *p53*-mediated growth arrest or apoptosis in the oncogene-expressing cells (30). An increased risk of SPM after index SCCHN was associated with each of $p14^{ARF}$ polymorphisms investigated in our previous study (19). The *MDM2*-rs2279744 T>G polymorphism creates a binding site for the

Table II. Association of individual polymorphisms of *p53*-related genes with SPM risk after index SCCHN

Polymorphisms	Total (n = 1283)		SPM free (n = 1163)		SPM (n = 120)		P[a]	HR (95% CI)[b]	P
	n	%	n	%	n	%			
p53									
WW	661	51.5	615	52.9	46	38.3	0.0024	1.0	0.009
WP/PP	622	48.5	548	47.1	74	61.7		1.6 (1.1–2.4)	
p73 rs2273953									
GA/AA	746	58.1	665	57.2	81	67.5	0.0291	1.0	0.035
GG	537	41.9	498	48.2	39	32.5		1.5 (1.0–2.2)	
$p14^{ARF}$-rs3731217									
TT	963	75.1	880	75.7	83	69.2	0.1172	1.0	0.032
TG/GG	320	24.9	283	24.3	37	30.8		1.5 (1.0–2.3)	
$p14^{ARF}$-rs3088440									
GG	1034	80.6	951	81.8	83	69.2	0.0009	1.0	0.026
GA/AA	249	19.4	212	18.2	37	30.8		1.6 (1.1–2.4)	
MDM2-rs2279744									
TT	741	57.7	656	56.4	85	70.8	0.0023	1.0	0.002
GT/GG	542	42.3	507	43.6	35	29.2		1.9 (1.2–2.7)	
MDM2-rs937283									
AA	343	26.7	316	27.2	27	22.5	0.2710	1.0	0.461
AG/GG	940	73.7	847	72.8	93	77.5		1.2 (0.8–1.8)	
MDM4-rs11801299									
GG	835	65.1	757	65.1	78	65.0	0.9842	1.0	0.956
AG/AA	448	34.9	406	34.9	42	35.0		1.1 (0.7–1.5)	
MDM4-rs1380576									
CC	547	42.6	500	43.0	47	39.2	0.4198	1.0	0.492
CG/GG	736	57.4	663	57.0	73	60.8		1.1 (0.8–1.6)	
MDM4-rs10900598									
GT/TT	883	68.8	810	69.7	73	60.8	0.0472	1.0	0.048
GG	400	31.2	353	30.3	47	39.2		1.4 (1.0–2.1)	

[a]Chi-square test for differences in the distribution of genotypes in *p53*-related genes between the patients who developed SPM and the patients who did not.
[b]Adjusted for age, sex, ethnicity, tobacco smoking and alcohol drinking in a Cox model.

Table III. Association of combined risk genotypes of *p53*-related genes with SPM risk after index SCCHN

	Total (n = 1283)		SPM free (n = 1163)		SPM (n = 120)		P[a]	HR (95% CI)[b]	P
	n	%	n	%	n	%			
No. of risk genotypes									
0–3 risk genotypes	463	36.1	436	37.5	27	22.5	<0.001	1.0	
4 risk genotypes	353	27.5	325	27.9	28	23.3		1.5 (0.9–2.5)	0.155
5 risk genotypes	266	20.7	238	20.5	28	23.3		1.8 (1.1–3.1)	0.025
6 risk genotypes	141	11.0	122	10.5	19	15.9		2.1 (1.2–3.8)	0.015
7 risk genotypes	49	3.8	35	3.0	14	11.7		5.5 (2.8–10.9)	<0.0001
8–9 risk genotypes	11	0.9	7	0.6	4	3.3		7.1 (2.4–21.0)	0.0004
Trend								<0.001	
Combined risk genotypes[c]									
Low-risk group	463	36.1	436	37.5	27	22.5	<0.001	1.0	
Medium-risk group	619	48.2	563	48.4	56	46.7		1.6 (1.0–2.6)	0.039
High-risk group	201	15.7	164	14.1	37	30.8		3.0 (1.8–5.0)	<0.0001
Trend								<0.001	

[a]Chi-square test for differences in the distribution of genotypes in *p53*-related genes between the patients who developed SPM and the patients who did not.
[b]Adjusted for age, sex, ethnicity, tobacco smoking and alcohol drinking in a Cox model.
[c]Low-risk group, individuals carrying 0–3 risk genotypes; medium-risk group, individuals carrying 4–5 risk genotypes; high-risk group, individuals carrying 6–9 risk genotypes.

common transcription factor Sp1, leading to low expression of p53 (31). Like *MDM2* that includes the *p53*-binding domain, a zinc finger motif and a C-terminal RING finger domain, *MDM4*, a structural homolog of *MDM2*, can also bind to *p53* and inhibit its ability to transactivate gene expression (32). Since most of these variants are low-penetrance polymorphisms that confer a minor risk, the combination of *p53*-related gene polymorphisms could result in more complete and accurate estimates of risk of SPM than that from a single variant (33).

Although the functional relevance of these variants except *p53* R72P and *MDM2*-rs2279744 (also called *MDM2* SNP309) has not yet been clarified, some biologically plausible information from public data may provide additional evidence to support our observed associations. We performed LD analysis from the recently released public information on 1000 genome data (March 2012) and used the SNPinfo tool (FuncPred, NIEHS, NIH, 2012) to predict relevant functionality of other seven variants. Our results indicated that all of these variants are potentially functional or highly in LD ($r^2 > 0.8$) with other nearby functional variants. *p73* G>A (rs2273953) is completely in LD ($r^2 = 1.0$) with another SNP rs1801173, which is located at the exonic splicing enhancer and might affect the splicing process. $p14^{ARF}$-rs3731217 is highly in LD with another variant, $p14^{ARF}$

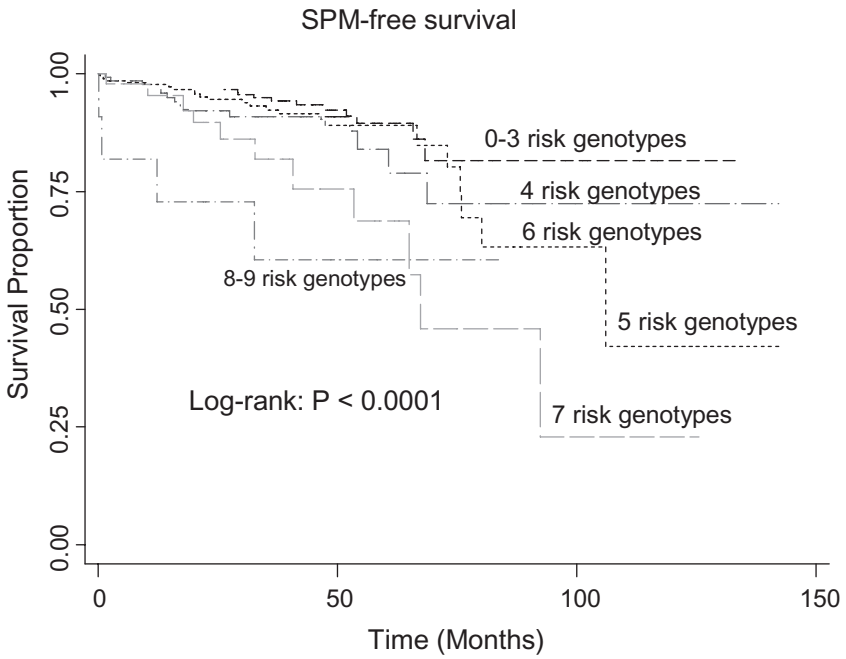

Fig. 1. SPM-free survival stratified by number of risk genotypes of *p53*-related genes.

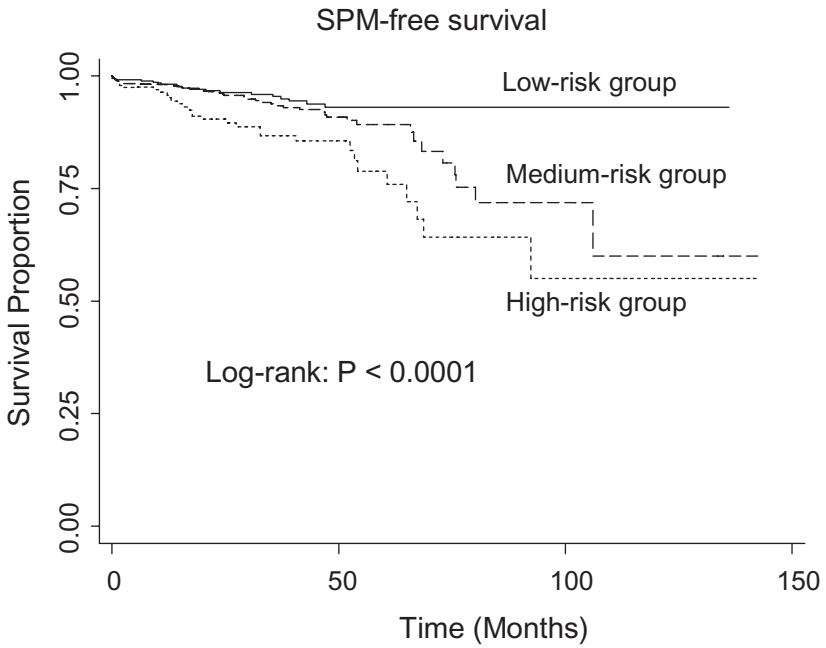

Fig. 2. SPM-free survival by the combined risk genotypes of *p53*-related genes in three risk groups.

rs2811711, which may influence the binding activity of transcription factors, whereas $p14^{ARF}$-rs3088440 in the 3′-UTR of $p14^{ARF}$ is within the putative binding sites of several micro-RNAs (e.g. *hsa-miR-328*, *hsa-miR-1291* and *has-miR-663b*). All three *MDM4* variants in this study were found to be highly in LD with other functional variants. For example, *MDM4*-rs1380576 is highly in LD with several other variants in the vicinity of this candidate variant to influence the binding activity of transcription factors (e.g. rs11240754, rs11240755, rs7367519, rs2926533, rs12032733, rs12738124 and rs4951077), the micro-RNA binding (e.g. rs4245739) and exonic splicing process (e.g. rs4245738). *MDM4*-rs11801299 is highly in LD with variant rs12028476, which is located at the 5′ flanking of *MDM4* and may have the potential to influence the binding of transcription factors. Finally, *MDM4*-rs10900598 is highly in LD with two variants, rs12034564 and rs4252745, and these two variants may, respectively, influence the binding activities of transcription factors or micro-RNAs (*hsa-miR-425* and *hsa-miR-542-3p*). Taken together, such information may additionally support our observed associations, which to some extent may be driven by limited study power due to small sample sizes in SPM events.

In our analysis of 1283 SCCHN patients, we analyzed the combined effects of the nine well-studied polymorphisms in *p53*-related genes on genetic susceptibility to SPM. Results showed that these variants jointly and significantly increased the risk of SPM with index

Table IV. Stratified analysis of combined risk genotypes of *p53*-related genes by smoking, index tumor site and SPM type

Variables	Low-risk group (reference)			Medium-risk group			*P*	High-risk group			*P*
	SPM free	SPM	HR (95% CI)[a]	SPM free	SPM	HR (95% CI)[a]		SPM free	SPM	HR (95% CI)[a]	
Age (median = 57 years)											
≤Median	228 (36.6)	8 (20.5)	1.0	313 (50.2)	19 (48.7)	1.9 (0.8–4.4)	0.122	82 (13.2)	12 (30.8)	4.2 (1.7–10.4)	0.002
>Median	208 (38.5)	19 (23.5)	1.0	250 (46.3)	37 (45.7)	1.5 (0.9–2.7)	0.143	82 (15.2)	25 (30.9)	2.3 (1.3–4.4)	0.007
Smoking											
Never	114 (35.7)	6 (24.0)	1.0	158 (49.6)	10 (40.0)	1.2 (0.4–3.5)	0.691	47 (14.7)	9 (36.0)	2.3 (0.8–6.8)	0.142
Ever	322 (38.2)	21 (22.1)	1.0	405 (48.0)	46 (48.4)	1.7 (1.0–2.9)	0.045	117 (13.8)	28 (29.5)	3.0 (1.7–5.4)	0.0002
Index tumor site											
Oro.	203 (38.7)	16 (33.3)	1.0	249 (47.6)	20 (41.7)	1.1 (0.6–2.1)	0.723	72 (13.7)	12 (25.0)	1.7 (0.8–3.6)	0.180
Non-oro.	233 (36.5)	11 (15.3)	1.0	314 (49.1)	36 (50.0)	2.4 (1.2–4.8)	0.010	92 (14.4)	25 (34.7)	5.0 (2.4–10.5)	<0.0001
SPM type											
TAS	436 (37.5)	19 (22.4)	1.0	563 (48.4)	41 (48.2)	1.7 (0.9–3.0)	0.052	164 (14.1)	25 (29.4)	3.0 (1.6–5.5)	0.0005
Non-TAS	436 (37.5)	8 (22.9)	1.0	563 (48.4)	15 (42.9)	1.7 (0.7–3.9)	0.250	164 (14.1)	12 (34.2)	3.3 (1.3–8.3)	0.010

Oro.: oropharyngeal cancer; TAS: tobacco-associated SPM.
[a]Adjusted for age, sex, ethnicity, tobacco smoking and alcohol drinking in a Cox model.

SCCHN, and an increasing number of putative risk genotypes was associated with increasing risk of SPM. These results support the notion that the development of SPM following index SCCHN may be a polygenic process, and risk evaluation in a pathway-based approach may yield higher predictive estimates of association (34).

We further performed a stratified analysis of the effects of combined low-risk, medium-risk and high-risk genotypes of these nine polymorphisms on risk of SPM among several subgroups. Although we found that young patients had a higher risk of SPM than the older patients in the high-risk group, the interaction of age with the combined risk genotypes was not statistically significant. Therefore, the residue confounding effect or bias caused by age in estimates of association in this study might not be severe. We found that significantly increased risk of SPM associated with the combined risk genotypes was confined to smokers and patients with index non-oropharyngeal cancers. Epidemiological studies have consistently demonstrated that most non-oropharyngeal cancers are associated with tobacco and alcohol use, whereas most oropharyngeal cancers are etiologically associated with human papillomavirus infection (35,36). The risk of developing SPMs in the aerodigestive tract is clearly associated with tobacco use before the diagnosis of the index tumor (5–7). Hence, it is biologically plausible that the risk of SPM in patients with index non-oropharyngeal cancers is likely attributable to tobacco use before and after diagnosis of the primary tumor.

The etiological concept of field cancerization might help to explain why patients with SCCHN may have a high possibility of developing SPM (37). Field cancerization, in which environmental carcinogens, such as tobacco, induce a field of the mucosa afflicted with premalignant lesions, may elevate risk of developing epithelial cancer throughout the upper aerodigestive tract (38). It was shown that these unresected fields contributed to SPM occurrence in patients treated for SCCHN (39). Our findings in index non-oropharyngeal cancers suggest that genetic variants in *p53*-related genes could modulate tobacco-induced development of SPM, possibly through gene–environment interactions.

Another finding in the present study was that the pronounced association between the combined risk genotypes and SPM risk was similar for all anatomical sites of SPM. Our results also showed that the high-risk group was significantly associated with the development of SPMs after index SCCHN in both tobacco-associated sites and non-tobacco-associated sites, suggesting that these polymorphisms of *p53*-related genes may play roles in both smoking-driven and non-smoking-induced SPMs. Although our results were significant in several subgroups, the insufficient numbers of SPMs in each subgroup, especially when combining the nine polymorphisms, may have limited the study power to detect a weak association. Therefore, larger sample sizes and well-characterized studies are needed to validate these results.

The present study has the following limitations. (i) Possible selection bias could not be ruled out because of the hospital-based study design. (ii) Approximately 85% of the patients were non-Hispanic whites, despite a large cohort of SCCHN patients. The results may, therefore, not be applicable to all ethnic populations. (iii) Clinical outcomes such as SPM were collected retrospectively, but the demographic, carcinogen exposure and clinical data for the cohort were collected prospectively. (iv) We did not have information on human papillomavirus infection or on continued smoking behavior after the index SCCHN diagnosis because of the retrospective study design. (v) The patients may not have had enough time to develop SPM or could have been lost to follow-up because the high proportion of never smokers and late stage index cancer patients as well as our strict criteria for determining SPM resulted in a SPM rate that was lower than expected. Therefore, the low rate of SPM and short follow-up time of study patients limited statistical power for the analysis, particularly for the stratified analysis. It is highly likely that most of these significant associations could be false positive results. Thus, our results could be chance findings and should be confirmed in larger studies.

Thus, our future studies will incorporate information such as human papillomavirus tumor status and continued smoking behavior. Finally, the small sample sizes of SPM events should be noted when interpreting the results as the small sample size increases the possibility that the statistically significant results could be due to chance. SPM rate (9.3%) in this study was lower than expected, and the SPM sample size limits statistical power in the subgroup analyses. Such low SPM rate may have been limited by short duration of follow-up for SPM development during the study period as the large proportion of study patients had a stage III or IV index cancer (75%) who were lost to follow-up, with only 120 patients having developed SPM, thus limiting statistical power to detect modest associations. Furthermore, the high prevalence (~27%) of never smokers and our strict definition of SPM may have also limited the observed incidence of SPM in this patient cohort. All of these limitations may have biased the observed association. Therefore, our findings are preliminary, and future prospective studies with larger sample sizes of SPM events and longer follow-up time in different populations are warranted to validate the results in multi-institutional groups such as INHANCE, the International Head and Neck Cancer Epidemiology Consortium.

In conclusion, the present study provides epidemiologic support for the combined effects of genetic variants of *p53*-related genes on risk of SPM in patients after index SCCHN. We also noted that the combined genetic effects were higher in ever smokers than in never smokers and in patients with index non-oropharyngeal cancers than with oropharyngeal cancers. The value of examining multiple polymorphisms simultaneously in genes involved in the same pathway is highlighted by our approach, which may improve the precision of risk estimates.

Funding

National Institute of Environmental Health Sciences (R01 ES-11740 to Q.W.); National Institutes of Health (P-30 CA-16672 to The University of Texas MD Anderson Cancer Center); National Institutes of Health (CA135679 and CA133099 to G.L.)

Acknowledgements

The authors wish to thank Ms Margaret Lung, Ms Angeli Fairley, Ms Liliana Mugartegui and Ms Kathryn Tipton for their assistance with patient recruitment. The authors gratefully thank Dawn Chalaire for editing the article.

Conflict of Interest Statement: None declared.

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Received December 10, 2012; revised February 14, 2013; accepted March 14, 2013