Published in final edited form as:

Mol Carcinog. 2011 September ; 50(9): 697–706. doi:10.1002/mc.20806.

Effects of *MDM2* promoter polymorphisms and *p53* codon 72 polymorphism on risk and age at onset of squamous cell carcinoma of the head and neck

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Abstract

Both *p53* tumor suppressor and murine double minute 2 (*MDM2*) oncoprotein are crucial in carcinogenesis. We hypothesized that *MDM2* promoter single nucleotide polymorphism (SNP)309, A2164G, and *p53* codon 72 SNP are associated with risk and age at onset of squamous cell carcinoma of head and neck (SCCHN). We genotyped these SNPs in a study of 1,083 Caucasian SCCHN cases and 1,090 cancer-free controls. Although none of these SNPs individually had a significant effect on risk of SCCHN, nor did their combined putative risk genotypes (i.e. *MDM2* SNP309 GT + GG, 2164 AA, and *p53* codon 72 CC), we found that individuals with 2–3 risk genotypes had significantly increased risk of non-opharyngeal cancer (OR = 1.42; 95% CI=1.07–1.88). This increased risk was more pronounced among young subjects, men, smokers, and drinkers. In addition, female patients carrying the *MDM2* SNP309 GT and GG genotypes showed a 3-year (56.7 years) and 9-year (51.2 years) earlier age at onset of non-opharyngeal cancer ($P_{\text{trend}} = 0.007$), respectively, compared with those carrying the TT genotype (60.1 years). The youngest age (42.5 years) at onset of non-opharyngeal cancer was observed in female patients with the combined *MDM2* SNP309 GG and *p53* codon 72 CC genotypes. The findings suggest that *MDM2* SNP309, A2164G, and *p53* codon 72 SNPs may collectively contribute to non-opharyngeal cancer risk and that *MDM2* SNP309 individually or in combination with *p53* codon 72 may accelerate the development of non-opharyngeal cancer in women. Further studies with large sample sizes are warranted to validate these results.

Keywords

squamous cell carcinoma of the head and neck; *MDM2*; *p53*; polymorphism; risk; age at onset

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Introduction

Squamous cell carcinomas of the head and neck (SCCHN) including cancers of the oral cavity, pharynx, and larynx are the most common malignancies in the world, representing more than 90% of all head and neck cancers. Although tobacco use and alcohol consumption have been well recognized as the major risk factors for SCCHN, only a fraction of individuals exposed to tobacco or alcohol develop SCCHN, suggesting that genetic factors may also play an important role in the etiology of SCCHN.

The p53 tumor suppressor plays a key role in organizing cellular responses to various types of stresses, including DNA damage and oncogene activation with apoptosis, cell cycle arrest, senescence, DNA repair, cell metabolism or autophagy [1–3]. Malfunction and mutations of *p53* have been found in most of human cancers, leading to deregulated p53 activity that allows to proliferate and survive unchecked [4]. The activity of p53 is regulated by many important proteins, and one of the most extensively studied regulators of p53 is the murine double minute 2 (MDM2) oncoprotein. MDM2 regulates the activity of p53 in at least three ways [5–8]. Firstly, MDM2 directly binds to the p53 transactivation domain, thus inhibiting its transcriptional activity. Secondly, MDM2 promotes ubiquitin-dependent proteasomal degradation of p53 by functioning as an E3 ubiquitin ligase. Finally, MDM2 shuttles p53 out of the nucleus to the cytoplasm of the cell, promoting the degradation of p53. Importantly, MDM2 forms a negative-feedback loop in regulating p53 activity, in which p53 induces transcription of *MDM2*, and, in turn, the MDM2 protein inhibits p53 activity [5].

Because MDM2 is a key negative regulator of p53 activity, over-expression of *MDM2* results in the inhibition of p53-mediated-transcriptional activation, thereby contributing to human carcinogenesis [9,10]. In fact, over-expression and amplification *MDM2* commonly occur in a wide variety of human cancers including SCCHN [11–16]. It has been reported that *MDM2* transgenic mice expressing approximately four times higher levels of MDM2 in various tissues compared to non-transgenic mice all develop spontaneous tumors, suggesting that over-expression of MDM2 affect tumor susceptibility in mice [9,10]. Taken together, *MDM2* may play a critical role in carcinogenesis, and therefore it is biologically plausible that functional genetic variants in the p53 pathway may have an effect on cancer development in the general population.

It has been reported that a T to G change of a single-nucleotide polymorphism (SNP) in the Sp1-binding site within the intronic promoter region of *MDM2* (SNP309, rs2279744) may increase the affinity of the Sp1 transcriptional factor, which results in higher levels of MDM2 mRNA and protein and attenuates the p53 pathway, both *in vitro* and *in vivo*, thereby increasing tumor susceptibility [10,17]. Consistently, the *MDM2* SNP309 GG genotype was found to be associated with at least an average nine-year earlier onset of disease in both hereditary and sporadic cancers in humans [16]. Likewise, a common SNP at codon 72 in exon 4 of *p53* (codon 72; rs1042522; G > C) has also been found to be of functional significance, with the C-allele having reduced apoptotic potential compared to the G-allele, which may modulate cancer risk, progression and/or response to treatment [11,12]. However, in a preliminary study with 304 cases and 333 cancer-free controls and a following-up study with 1,111 cases and 1,130 cases, we did not find an association between *p53* codon 72 SNP and SCCHN risk [13,14], although the *p53* codon 72 C-allele was initially found to be associated with an earlier age of onset of SCCHN [13]. These early studies inspired us to further investigate a possible synergistic role of SNPs in *MDM2* and *p53* in modulating risk and age of SCCHN onset. It is known that gene regulatory regions, such as the promoter regions, are important to gene function through binding to specific transcription factors and regulating the initiation of gene transcription [15]. Since p53 and

MDM2 physically and functionally interact in the p53 pathway, we selected the *MDM2* promoter SNPs (SNP309 and A2164G) together with the well-studied *p53* codon 72 (rs1042522) SNP to test our hypothesis that these SNPs, through synergistic effects, are associated with risk and age at onset of SCCHN.

Materials and Methods

Patients and controls

The study population included 1,083 patients with newly diagnosed SCCHN between October 1999 and October 2007, including cancers of the oral cavity, oropharynx, hypopharynx and larynx, identified at The University of Texas M. D. Anderson Cancer Center. Patients with second primary tumors, primary tumors of the skin, nasopharynx, sinonasal tract, and/or any histopathologic diagnoses other than squamous cell carcinoma were excluded. All patients were Caucasians and had not received any treatment at the time of recruitment. Approximately 93% of the eligible patients contacted chose to participate in this study. The 1,090 cancer-free controls we recruited in the same period were genetically unrelated visitors or companions of patients seen at The M. D. Anderson Cancer Center clinics and were frequency matched to the cases by age (± 5 years), sex and ethnicity. The response rate of the eligible controls was approximately 85%. Having signed a written informed consent during the interview, all subjects enrolled in the study were interviewed to gather demographic data and history of tobacco and alcohol use. Each subject donated one-time 30-mL of blood, of which 1 ml was used for genomic DNA extraction with a DNA blood Mini Kit (Qiagen, Valencia, CA) according to the manufacture's instructions. The research protocol was approved by the M.D. Anderson Institutional Review Board.

Genotyping

Genotyping data of *p53* codon 72 polymorphism were available from our previously published study [14]. The polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assay for the *p53* codon 72 polymorphism has been described elsewhere [13].

Except for the *MDM2* SNP309, we also identified another three SNPs, i.e., G1797C (rs937282), A2164G (rs937283), and C2326 T (rs2870820) that have a minor allele frequency (MAF) > 10% within the promoter region of *MDM2* in a published SNP database (<http://egp.gs.washington.edu/data/mdm2/mdm2x.csnp.txt>). The linkage disequilibrium (LD) analysis by the computational tool of LD Tag SNP Selection (Tag SNP) (<http://manticore.niehs.nih.gov/snptag.htm>) revealed that rs937283 (A2164G) tags both rs937282 (G1797C) and rs2870820 (C2326T) with LD coefficient $r^2 = 0.90$ in the European population of the NCBI/dbSNP database. Thus, two *MDM2* promoter SNPs, i.e., *MDM2* SNP309 and A2164, were selected to genotype in the present study.

MDM2 SNP309 was genotyped by the PCR- RFLP assay. The primers used in the PCR-RFLP for *MDM2* SNP309 was: (forward) 5'-CGGGAGTTCAGGGTAAAGGT -3' and (reverse) 5'-AGCAAGTCGGTGCTT ACCTG -3'. The PCR reactions profile consisted of an initial melting step of 95°C of 5 min, 35 cycles of 95°C for 30s, 55°C for 45s and 72°C for 1 min and a final extension step of 72°C for 10 min. The 352-bp PCR product was digested with *MspA1* I (New England Biolabs, Beverly, MA) at 37°C overnight. The wild-type allele (TT) produced three bands of 233, 88, and 31bp; wild-type/variant allele (TG) produced 233, 187, 88, 46 and 31bp and the variant allele (GG) produced 187, 88, 46 and 31bp. PCR-RFLP was conducted and the results were evaluated without knowledge of the subjects' case-control status. Approximately 10% of the samples were randomly selected and repeated with PCR-RFLP, and the results were 100% concordant.

MDM2 A2164G (rs937283) was genotyped using the Applied Biosystems TaqMan genotyping platform according to the manufacture's recommendations. Briefly, the reactions were prepared by using TaqMan Universal Master Mix, 40×SNP Genotyping Assay Mix, DNase-free water, and 5-ng genomic DNA in a final volume of 5 µL per reaction. Both negative and positive controls and three samples with known genotypes were included in each plate to ensure the accuracy of the genotyping. The PCR amplification was run and the plate was read using a TaqMan 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). The analyzed fluorescence results were then auto-called into the genotypes using the built-in SDS2.3 software of the system.

Statistical Analysis

The differences in the distributions of categorical variables, including demographic characteristics, tobacco smoking and alcohol use, and genotype/allele frequencies of selected SNPs between the patients and controls were evaluated by Chi-square tests. The deviation from Hardy-Weinberg equilibrium was tested by chi-square goodness-of-fit test. Crude or adjusted (for age, sex, smoking and drinking status) odds ratios (ORs) and 95% confidence intervals (CIs) were obtained from unconditional univariate and multivariable logistic regression analyses to evaluate associations between SNPs and SCCHN risk in the case-control analysis, which was further stratified by age, sex, smoking and drinking status. Student *t*-test and general linear models were used to evaluate the differences in age at onset between different genotype groups in patients and the trend in association with the age at onset, respectively. Finally, we calculated the false positive report probability (FPRP) for observed statistically significant associations in a range of prior probabilities, and used a threshold of noteworthiness of FPRP ≤0.2 in the present study[18]. All statistics tests were two-sided with a 0.05 significance level and all data were analyzed with SAS statistical software (SAS version 9.2; SAS Institute Inc., Cary, NC).

Results

Characteristics of the study population

In this study, 1,083 SCCHN patients and 1,090 controls of Caucasians were included from our ongoing studies whose DNA samples were available. The frequency distribution of selected characteristics of the patients and controls is presented in Table 1. Because of frequency matching by design, there was no statistical difference in the distributions of age and sex between cases and controls: The mean age was 57.1 years for the cases (± 11.2 years; range, 18–90 years) and 56.7 years for the controls (± 11.0 years; range, 20–87 years) ($P = 0.615$), and 75.2% and 24.8% of the cases and 76.3% and 23.7% of the controls were men and women, respectively ($P = 0.525$). However, the cases were more likely to be smokers and drinkers than the controls ($P < 0.001$ for both). Therefore, smoking and drinking were adjusted in the subsequent multivariate logistic regression analyses. Of the 1,083 cases, 318 (29.3%) had cancer of the oral cavity, 550 (50.8%) of oropharynx, 43 (4.0%) of hypopharynx and 172 (15.9%) of larynx.

Genotypes distribution of polymorphisms of *MDM2* and *p53* codon 72 polymorphism between cases and controls

The allele and genotype distributions of the studied SNPs in cases and controls and their associations with SCCHN risk are summarized in Table 2. All observed genotype distributions among controls were in agreement with the Hardy-Weinberg equilibrium (all $P > 0.05$). Linkage disequilibrium (LD) analysis revealed that the minor variant alleles of *MDM2* SNP309 and *MDM2* A2164G were not in LD ($r^2 = 0.29$ and $D' = 0.88$) among the controls. No significant difference in the genotype frequencies of the studied SNPs was found between the cases and controls ($P = 0.638$ for *MDM2* SNP309, $P = 0.580$ for *MDM2*

A2164G and $P = 0.193$ for *p53* codon 72). Although none of the variant genotypes alone was associated with significantly altered risk, the *MDM2* SNP309 GT and GG genotypes tended to be associated with non-significantly increased SCCHN risk (adjusted OR = 1.11; 95% CI = 0.92–1.34 for GT, and adjusted OR = 1.08; 95% CI = 0.81–1.43 GG), the AG and GG genotypes of *MDM2* A2164G tended to be associated with non-significantly reduced SCCHN risk (OR = 0.98; 95% CI = 0.81–1.20 for AG, and OR = 0.83; 95% CI = 0.64–1.07 for GG), and the *p53* codon 72 CC genotype tended to be associated with non-significantly increased SCCHN risk (adjusted OR = 1.30; 95% CI = 0.91–1.86). In the haplotype analysis of *MDM2* SNP309 and *MDM2* A2164G, we did not observe any risk of SCCHN associated with specific common haplotypes (frequencies > 5%) of these two SNPs (data not shown). In addition, we performed a meta-analysis of *MDM2* SNP309 with our and other six published studies of 1595 cases and 2254 controls (Figure 1). Consistently, we found that the *MDM2* SNP309 was not significantly associated with risk of SCCHN (the pooled OR = 1.05; 95% CI = 0.93–1.18 for GT/GG genotype; $P_{\text{heterogeneity}} = 0.69$).

Considering a possible combined effect from the two SNPs in *MDM2* and *p53* codon 72 SNP on risk of SCCHN, we combined them by the number of the putative risk genotypes (i.e., *MDM2* SNP309 GT/TT, *MDM2* 2164 AA, and *p53* codon72 CC) to assess their possible combined effect on risk of SCCHN, and we did not find the combined risk genotypes were statistically associated with increased overall risk of SCCHN (Table 2).

Association and stratification analysis between the combined genotypes of *MDM2* polymorphisms and *p53* codon 72 polymorphism and risk of non-oro-pharyngeal cancer

Because SCCHN is a heterogeneous group of tumors with different primary sites, we further evaluated the combined effect of polymorphisms of *MDM2* SNP309, A2164G and *p53* codon 72 on the risk of SCCHN stratified by tumor site. As shown in Table 2, the combined risk genotypes were found to be associated with increased risk of SCCHN at non-oro-pharyngeal sites but not at oro-pharyngeal site. There were more individuals with two or three risk genotypes and fewer individuals with one risk genotype among the cases of non-oro-pharyngeal cancer (35.1% and 32.0%, respectively) than among the controls (27.5% and 37.6%, respectively), and these differences were statistically significant ($P = 0.005$). The combined genotype with 2–3 risk genotypes was associated a statistically significantly increased risk of non-oro-pharyngeal cancer compared with those with 0 risk genotypes (OR = 1.42; 95% CI = 1.07–1.88) after adjustment for age, sex, smoking, and drinking status. In the further stratified analysis by age, sex, smoking and drinking status, an increased risk associated with the combined genotype with 2–3 risk genotypes was more pronounced in younger subjects (≤ 57 years old) (OR = 1.61; 95% CI = 1.05–2.47), men (OR = 1.41; 95% CI = 1.01–1.98), ever smokers (OR = 1.43; 95% CI = 1.02–2.02) and ever drinkers (OR = 1.47; 95% CI = 1.03–2.11). However, there was no statistical evidence for interactions among these variables in further multivariate logistic regression models (data not shown).

Finally, because multiple tests had been performed, we estimated the false positive reporting probability (FPRP) for all the statistically significant results of non-oro-pharyngeal cancer. As shown in Table 4, the association of the combined genotype with 2–3 risk genotypes with the risk of non-oro-pharyngeal cancer yielded an FPRP value of 0.188 for a prior probability of 0.1, assuming that the OR for non-oro-pharyngeal cancer risk was 1.50 and had a statistical power of 0.78, which was a noteworthy finding. The FPRP values for a prior probability of 0.1 for the subgroup analyses by age, sex, smoking status and drinking status was 0.444, 0.311, 0.353 and 0.334, respectively, assuming that the OR for the combined genotype was 1.50 with a post-hoc statistical power of 0.54, 0.73, 0.67, and 0.68, respectively, suggesting some possible biases in these findings.

Associations among polymorphisms of *MDM2* and *p53* codon 72 and age at onset of SCCHN

We further evaluated age at onset of SCCHN by *MDM2* SNPs and *p53* codon 72 SNP in the case-only analysis with 1083 patients. The mean ages of SCCHN onset for the TT, TG, and GG genotypes of *MDM2* SNP309 were 57.7 (± 11.0), 56.8 (± 11.0), and 56.3 (± 12.2) years old, respectively. These differences in age of disease onset were not significantly different. Similar results were also shown for *MDM2* A2164G and *p53* codon 72 (data not shown). Then we further evaluated age at onset of oropharyngeal cancer (550 patients) and non-oropharyngeal cancer (533 patients) for these three SNPs, respectively, and no statistically significant association with age at onset were observed (data not shown). However, there were distinct differences in age at onset of non-oropharyngeal cancer between sexes.

We found that *MDM2* SNP309 genotype was associated with an earlier age at onset of non-oropharyngeal cancer in an allele-dependent manner (i.e., a younger age at onset with increasing number of G alleles, $P_{\text{trend}} = 0.007$, Figure 2A). Specifically, female patients with TG and GG genotypes showed on average a 3-year (56.7 years) and 9-year (51.2 years) earlier age at onset of non-oropharyngeal cancer, respectively, compared to those with the wild-type TT (60.1 years). However, such findings were not observed in male patients (data not shown). When the genotypes of *MDM2* SNP 309 and *p53* codon 72 SNP were combined, there was also a trend of age at onset in a genotype-dependent manner ($P_{\text{trend}} = 0.004$, Figure 2B), which was not observed in male patients (data not shown). Notably, female patients with the combined *MDM2* SNP309 GG and *p53* codon 72 CC genotypes had the youngest age at onset (42.5 years), compared with those with the combined *MDM2* TT and *p53* codon 72 GG/CG genotypes (60.4 years). However, these differences in female patients were not observed for *MDM2* A2164G SNP alone or in combination with *p53* codon 72 SNP (data not shown).

Discussion

In a study population of 1,083 SCCHN patients and 1,090 cancer-free controls of Caucasians, although none of the three SNPs in *MDM2* and *p53* (*MDM2* SNP309, A2164G, and *p53* codon 72) was alone associated with risk of overall SCCHN, a joint effect of these three SNPs may contribute to risk of SCCHN at non-oropharyngeal sites. Furthermore, it appeared that the *MDM2* SNP309 G-allele was associated with an early age at onset of non-oropharyngeal cancer in an allele-dose response manner in female patients, and this age effect could be further amplified by the *p53* codon 72 C-allele. In contrast, we did not find similar effects for *MDM2* A2164G and *p53* codon 72, either individually or in combination. Given the role of MDM2 as a key inhibitor of p53, it is biologically plausible that these two SNPs in the *MDM2* promoter region and *p53* codon 72 SNP may collectively modulate risk of non-oropharyngeal cancer.

It had been demonstrated that the G-allele of SNP309 located in the promoter region of *MDM2* increases the affinity of the transcriptional activator Sp1, resulting in high levels of *MDM2* mRNA and MDM2 protein, thereby affecting p53 tumor suppressor activity and potentially cancer development in humans. Recently, in mouse models carrying either the polymorphic *MDM2*^{SNP309G} or *MDM2*^{SNP309T} allele [19], Post et al. found that *MDM2*^{SNP309G/G} cells exhibit elevated MDM2 levels, reduced p53 levels, and decreased p53-dependent apoptosis in response to DNA damage; importantly, those mice with two *MDM2*^{SNP309G} alleles have an attenuation of the p53 pathway resulting in a decreased latency to tumor formation and decreased survival. In addition, over-expression of MDM2 could be involved in downregulation of other important cellular proteins such as pRB, E2F1, and p19ARF, further promoting carcinogenesis [17,20]. Therefore, it is likely that *MDM2* SNP309 may be a rate-limiting event in carcinogenesis [16].

A number of studies have investigated associations between SNPs of *MDM2* SNP309 and *p53* codon 72 and SCCHN risk, but the results are controversial [12,16,21–27]. A recent meta-analysis reported no association between the *p53* codon 72 polymorphism and risk of oral carcinoma [22]. So far, six published studies with relatively small sample sizes have investigated the association between *MDM2* SNP309 and the risk or age at onset of SCCHN [28–33]. Our mini meta-analysis using these published data and ours in the present study showed that *MDM2* SNP309 was not significantly associated with risk of SCCHN. It is possible that the effect of the *MDM2* SNP309 on SCCHN risk may be modest and could not be detected in the present study, or the effect can be modified by other SNPs of other genes. Indeed, we did find that those who carried the two to three risk genotypes (i.e., *MDM2* SNP309 GT/TT, 2164 AA, and *p53* codon72 CC) appeared to have an increased risk of non-opharyngeal cancer, and this risk was more pronounced among ever smokers and ever drinkers. Human papillomavirus (HPV)-related SCCHN occurs predominantly in the oropharynx, and HPV is an established etiologic agent of oropharyngeal cancer. It has been reported that HPV-related oropharyngeal cancer is most likely among those who are less smoking and drinking. In contrast, non-HPV related SCCHN (oral cavity, hypopharyngeal, and larynx) is more commonly linked with heavy tobacco and alcohol use [34–36]. Hence, our findings in non-opharyngeal cancer suggest that genetic variants in *MDM2* and *P53* could modulate non-HPV-induced carcinogenesis through gene-environment interaction. However, we did not find statistical evidence for interactions among these variables in the current study, which could be possible that the limited sample size in the group of non-opharyngeal cancer (530 cases) may not have enough power to detect such interactions. Further studies with large sample size are warranted to confirm our findings in non-opharyngeal cancer.

Although *MDM2* SNP309 and *p53* codon 72 SNPs have been demonstrated to be functional, the function of *MDM2* promoter A2164G SNP has not yet been reported. *MDM2* A2164G has been predicted to be functional by the computation tool of SNP Function Prediction (FuncPred, <http://manticore.niehs.nih.gov/snpfunc.htm>). Hence, based on its putative function and our data reported herein, further functional studies need to be performed on *MDM2* A2164G and other untyped putative functional polymorphisms that are in LD with this SNP.

With respect to the association between *MDM2* SNP309 and the age at onset of SCCHN in female patients, two published studies found that the GG genotype was associated with an early age at onset of SCCHN in an Asian population. A Japanese case-only study with 119 SCCHN patients found that the SCCHN patients with the GG genotype had a nine-year earlier tumor onset in comparison with those with the TT genotype [29]. Huang *et al.* found that the *MDM2* SNP309 G-allele was associated with at least a three-year earlier age of tumor onset in a Taiwanese population [30]. In contrast, another study showed that patients with the GG genotype showed a five-year later age of tumor onset than those with the TT genotype in women in a Malaysian population [33]. In the current case-only analysis with 1083 SCCHN patients, we found that the *MDM2* SNP309 G-allele associated with an early age at onset of SCCHN at non-opharyngeal sites in female patients, which could be as early as 42.5 years old for women who also carried the *p53* codon 72 C-allele.

It is likely that primarily female-specific hormones, such as estrogen, could allow for the SNP309 G-allele to accelerate tumor formation in women [37,38]. More recent studies reported that estrogen receptors were expressed in the majority of SCCHN tumors and cell lines [39–41]. It had been shown that estrogen signaling could regulate *MDM2* expression levels [42,43]. SNP309 is located only 10 bp away from the potential estrogen receptor responsive region of the *MDM2* promoter. Since SNP309 enhances the binding affinity of Sp1, a co-transcriptional activator of hormone receptor, it may affect the transcriptional

regulation of *MDM2* by estrogen signaling. Indeed, Hu *et al.* demonstrated that estrogen preferentially stimulated transcription of the *MDM2* gene from the SNP309 G-allele and increased the levels of MDM2 protein in estrogen-responsive cells homozygous for SNP 309 (G/G), leading to further attenuation of the p53 pathway [42].

Although our results are somewhat inconsistent with those previously published findings on SCCHN [31–33], our findings are consistent with the biological function of the *MDM2* SNP309 SNP and similar to previous reports on other cancer types including colorectal cancer, diffuse large B-cell lymphoma, soft tissue sarcoma, breast cancer and ovarian cancer, in which acceleration of tumor formation was shown to be associated with the G-allele, particularly in female patients [26,37,38,44]. For example, Bond G. *et al.* also demonstrated that the GG genotype was associated with a 13-year, 14-year, and a 10-year earlier age at onset of diffuse large B-cell lymphoma, soft tissue sarcomas and colorectal cancer, respectively, only in women [37,38]. A Finland study found that GG carriers showed a 2.7-year earlier age at onset of colorectal cancer than TT carriers only in women but not in men [32]; however, that study found that *MDM2* SNP309 was neither associated with risk nor with the age at onset of SCCHN, and further stratified analysis by sex was not reported in that study because of its small sample size (157 SCCHN patients and 185 controls). The discrepancies between our present study and those reported studies could be due to differences in ethnicity and study designs. Indeed, genotype distributions of *MDM2* SNP309 may vary by ethnicity. For example, the frequencies of the GG, TG, and TT genotypes of *MDM2* SNP309 among our Caucasians population were 11.9%, 43.3%, and 44.8%, respectively, compared with 27.5%, 41.7%, and 30.8% of 120 Japanese subjects in the study by Nakashima *et al.* [29], and 27.1%, 51.3%, and 21.5% of 1,272 Taiwanese subjects in the study by Huang *et al.* [30]. Therefore, these ethnic variations in genotype distributions and their influence on the risk of cancer need to be further investigated.

To the best of our knowledge, the present study is the first one using a large patient cohort to investigate the association between the risk and age at onset of SCCHN and *MDM2* promoter SNPs, individually and in combination with *p53* codon 72 SNP. Furthermore, our study population is ethnically homogeneous for Caucasians, decreasing possible biases from population stratification. Nevertheless, some potential limitations in the current study should be considered. Firstly, three SNPs were included in the analyses and the possibility of false-positive associations could not be ruled out because of multiple tests. The FPRP analysis showed that some possible biases may exist in our findings. Secondly, although our sample size was relatively large, the small sample size in subgroup analyses may have limited statistical power. Hence, our findings need to be confirmed by studies with larger sample sizes. Finally, HPV is another etiologic factor, in addition to tobacco and alcohol, for SCCHN, particularly for oropharyngeal cancer. However, when we evaluated the modification effects of polymorphisms in *MDM2* and *p53* on the risk of SCCHN, HPV status was not taken into account because only a small subset of SCCHN patients included in this study had available data on HPV infection. This limitation should be overcome in our future studies. Despite these limitations, our findings are biologically plausible. Our results provide some novel clues for the role of *MDM2* and *p53* SNPs in the development of non-oropharyngeal cancer and age at onset of the disease.

In summary, we found that the combined risk genotypes of three SNPs in *MDM2* and *p53* (i.e., *MDM2* SNP309 GT+GG, 2164AA, and *p53* codon 72 CC) were significantly associated with risk of non-oropharyngeal cancer. We also found that the *MDM2* SNP309 G-allele was associated with an earlier age at onset of non-oropharyngeal cancer in women but not in men, and this age effect could be amplified by the *p53* codon 72 C-allele. Our findings indicate that *MDM2* promoter SNPs, i.e., *MDM2* SNP309 and *MDM2* A2164G, as well as *p53* codon 72 SNP may collectively contribute to the development of non-

oropharyngeal cancer and that *MDM2* SNP309 individually or in combination with *p53* codon 72 may accelerate non-oropharyngeal cancer development in women. Further independent studies with large sample sizes and relative functional studies are needed to validate these findings.

Acknowledgments

We thank Ana Neumann, Margaret Lung, Jessica Fiske and Shara Challa for their assistance in recruiting the subjects and gathering the questionnaire information, Yawei Qiao, Jianzhong He, Kejing Xu and Min Zhao for laboratory assistance, and Dakai Zhu for his technical support. This work was partly supported by the National Institute of Health grants R01 CA131274 and R01 ES011740 (Q. Wei), ES015587 (D.G. Johnson), P50 CA097007 (Scott Lippman), and P30 CA016672 (The University of Texas M. D. Anderson Cancer Center). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

Abbreviations

SCCHN	squamous cell carcinoma of head and neck cancer
SNP	single nucleotide polymorphism
OR	odds ratio
CI	confidence interval
PCR	PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism
LD	linkage disequilibrium

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Figure 1.

Figure 2A

Figure 2B

Figure 2.

Table 1

Demographic characteristics of SCCHN cases and cancer-free controls

Variables	Cases	Controls	<i>P</i> *
	No. (%)	No. (%)	
All subjects	1,083 (100%)	1,090 (100%)	
Age (years)			0.615
Range	18–90	20–87	
Mean	57.1 ± 11.2	56.7 ± 11.0	
≤ 57 †	578 (53.4)	570 (52.3)	
> 57	505 (46.6)	520 (47.7)	
Sex			0.525
Male	814 (75.2)	832 (76.3)	
Female	269 (24.8)	258 (23.7)	
Smoking Status			<0.0001
Never	302 (27.9)	535 (49.1)	
Former smoker	370 (34.2)	397 (36.4)	
Current smoker	411 (37.9)	158 (14.5)	
Alcohol use			<0.0001
Never	295 (27.2)	473 (43.4)	
Former	235 (21.7)	176 (16.1)	
Current	553 (51.1)	441 (40.5)	
Tumor Site			
Oropharynx	550 (50.8)	-	
Non-oropharynx	533 (49.2)	-	

* Two-sided chi-square test.

† The median age of the controls.

Genotypes and allele frequency of polymorphisms in *MDM2* and *P53* among cases and cancer-free controls and their associations with SCCHN risk

Table 2

Variant genotypes	Case (n = 1083)		Controls (n = 1090) *		P [†]	Adjusted OR (95% CI) [‡]
	n	%	n	%		
MDM2 SNP309 (T>G; rs2279744)						
TT	463	42.7	488	44.8	0.638	1.00
GT	486	44.9	472	43.3		1.11 (0.92–1.34)
GG	134	12.4	130	11.9		1.08 (0.81–1.43)
GT/GG	620	57.2	602	55.2		1.10 (0.92–1.32)
G allele frequency		34.9		33.6	0.392	
MDM2 A2164G (A>G;rs937283) [§]						
AA	369	34.2	355	32.6	0.580	1.00
AG	522	48.4	529	48.6		0.98 (0.81–1.20)
GG	187	17.4	205	18.8		0.83 (0.64–1.07)
AG/GG	709	65.8	734	67.4		0.95 (0.79–1.14)
G allele frequency		41.6		43.1	0.300	
p53 codon72 (rs1042522; G>C)						
GG	593	54.76	597	54.76	0.193	1.00
CG	405	37.40	428	39.27		0.95 (0.79–1.14)
CC	85	7.85	65	5.96		1.30 (0.91–1.86)
CG/CC	490	45.2	493	45.2		1.00 (0.83–1.19)
C allele frequency		28.5		25.6	0.476	
Number of combined risk genotypes of MDM2 polymorphisms and p53 codon 72 polymorphism//						
0	369	34.2	380	34.9	0.077	1.00
1	367	34.1	409	37.6		0.95 (0.77–1.17)
2–3	342	31.7	300	27.5		1.16 (0.93–1.45)
P _{trend}						0.207
Association of combined risk genotypes and SCCHN risk stratified by tumor sites						
oropharyngeal cancer						
Number of combined risk genotypes//						

Variant genotypes	Case (n = 1083)		Controls (n = 1090) [*]		<i>P</i> [†]	Adjusted OR (95% CI) [‡]
	n	%	n	%		
0	194	35.5	380	34.9	0.840	1.00
1	197	36.1	409	37.6		0.96 (0.75–1.23)
2–3	155	28.4	300	27.5		1.01 (0.78–1.32)
<i>P</i> _{trend}						0.945
non-oro-pharyngeal cancer						
Number of combined risk genotypes ^{//}						
0	175	32.9	380	34.9	0.005	1.00
1	170	32.0	409	37.6		0.93 (0.70–1.23)
2–3	187	35.1	300	27.5		1.42 (1.07–1.88)
<i>P</i> _{trend}						0.018

^{*}The observed genotype frequency among the control subjects was in agreement with the Hardy-Weinberg equilibrium (chi-square = 0.931, *P* = 0.335 for rs2279744, chi-square = 0.102, *P* = 0.750 for rs937283, and chi-square = 1.041, *P* = 0.308 for rs1042522).

[†]Two-sided χ^2 test for differences in distribution of genotypes between the cases and controls.

[‡]Obtained from logistic regression models with adjustment for age, sex, smoking status and alcohol use.

[§]5 cases and 1 control failed in the *MDM2* rs937283 genotyping assay.

^{//}This represents the number of risk genotypes; Assumed risk genotypes used for the calculation: *MDM2* rs2279744 GT/GG, *MDM2* rs937283 AA, and *p53* condon72 CC.

Table 3

Association and stratification analysis of the combined genotypes of *MDM2* polymorphisms and *p53* codon 72 polymorphism and the risk of non-opharyngeal cancer

Variables	n, case/control	Number of combined risk genotypes of rs2279744 and rs937283 in <i>MDM2</i> and <i>p53</i> codon72 [*]				P trend [‡]
		% (case/control)	Adjusted OR (95% CI) [‡]	Adjusted OR (95% CI) [‡]	Adjusted OR (95% CI) [‡]	
		0	1	2-3	0 verse 1	0 verse 2-3
Total	532/1089	32.9/34.9	31.9/37.6	35.2/27.5	0.93 (0.70-1.23)	1.42 (1.07-1.88)
Age (years)						
≤ 57	228/569	30.7/36.7	35.5/35.9	33.8/27.4	1.27 (0.84-1.91)	1.61 (1.05-2.47)
> 57	304/520	34.5/32.9	29.3/39.4	36.2/27.7	0.72 (0.49-1.05)	1.29 (0.88-1.90)
Sex						
Female	182/258	32.4/36.4	34.6/36.4	33.0/27.1	1.08 (0.65-1.78)	1.39 (0.83-2.35)
Male	350/831	33.1/34.4	30.6/37.9	36.3/27.7	0.89 (0.63-1.24)	1.41 (1.01-1.98)
Smoking status						
Never	102/535	32.4/33.8	33.3/38.5	34.3/27.7	0.95 (0.56-1.61)	1.36 (0.80-2.31)
Ever	430/554	33.0/35.9	31.6/36.6	35.4/27.4	0.92 (0.66-1.29)	1.43 (1.02-2.02)
Drinking status						
Never	154/473	31.2/35.7	38.9/36.4	29.9/27.9	1.46 (0.92-2.32)	1.37 (0.84-2.24)
Ever	378/616	33.6/34.3	29.1/38.5	37.3/27.3	0.74 (0.52-1.07)	1.47 (1.03-2.11)

^{*}This represents the number of risk alleles used in the calculation; Assumed risk genotypes used for the calculation: *MDM2* rs2279744 GT/GG, *MDM2* rs937283 AA, and *p53* codon72 CC.

[‡]Obtained from logistic regression model with adjustment for age, sex, smoking status and alcohol use.

False positive reporting probability values for associations between the combined genotypes of polymorphisms in *MDM2* and *p53* and non-oro-pharyngeal cancer risk

Table 4

0 risk genotype vs. 1–3 risk genotypes	Positive OR and 95% CI ^a	<i>p</i> ^b	Statistical power ^c	Prior probability				
				0.25	0.1	0.01	0.001	0.0001
All subjects	1.42 (1.07–1.88)	0.0202	0.78	0.0717	0.1881	0.7181	0.9626	0.9768
younger age (≤57)	1.61 (1.05–2.47)	0.0475	0.54	0.21	0.4437	0.8977	0.9888	0.9989
Male subjects	1.41 (1.01–1.98)	0.0477	0.73	0.1307	0.3108	0.8322	0.9804	0.998
Ever smokers	1.43 (1.02–2.02)	0.0334	0.67	0.1539	0.353	0.8572	0.9838	0.9984
Ever drinkers	1.47 (1.03–2.11)	0.0377	0.68	0.1433	0.3342	0.8467	0.9824	0.9982

^aThe adjusted OR

^bthe omnibus Chi-square test of the combination genotype distributions.

^cCalculated using study subjects to detect an OR of 1.5 with the common combined genotype used as the reference.