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Functional single nucleotide polymorphisms of the *RASSF3* gene and susceptibility to squamous cell carcinoma of the head and neck

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Abstract

Background—*RASSF3* suppresses tumour formation through uncertain mechanisms, but it is an important gene of p53-dependent apoptosis. *RASSF3* depletion impairs DNA repair after DNA damage, leading to polyploidy. The authors hypothesised that potential functional single-nucleotide polymorphisms (SNPs) of *RASSF3* are associated with risk of squamous cell carcinoma of the head and neck (SCCHN).

Methods—The authors used a functional SNP approach to evaluate the associations between common (minor allele frequency > 0.05), putative functional variants in *RASSF3* and risk of SCCHN. Four selected such functional SNPs (rs6581580 T>G, rs7313765 G>A, rs12311754 G>C and rs1147098 T>C) in *RASSF3* were identified and genotyped in 1087 patients and 1090 cancer-free controls in a non-Hispanic white population.

Results—The authors found that two SNPs were significantly associated with SCCHN risk. Carriers of the variant rs6581580G and rs7313765A alleles were at a reduced SCCHN risk, compared with the corresponding common homozygotes [adjusted odds ratio (OR) = 0.75 and 0.73 and 95% confidence interval (CI) = 0.62–0.91 and 0.60–0.88, respectively, for dominant models; and P_{trend} = 0.012 and 0.041, respectively, for additive models], particularly for non-opharyngeal tumours (adjusted OR = 0.68 and 0.60 and 95% CI = 0.53–0.86 and 0.47–0.77, respectively, for dominant models). In the genotype–phenotype correlation analysis of peripheral blood mononuclear cells from 102 cancer-free controls, the rs6581580 GG genotype was associated with significantly increased expression levels of *RASSF3* mRNA (P = 0.038), compared with the TT genotype. Additional functional experiments further showed that variant G allele of rs6581580 had a significantly stronger binding affinity to the nuclear protein extracts than the T allele.

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Conflict of interest statement

None declared.

Conclusion—Taken together, these findings indicate that the *RASSF3* promoter rs6581580 T>G SNP is potentially functional, modulating susceptibility to SCCHN among non-Hispanic whites. Larger replication studies are needed to confirm our findings.

Keywords

RASSF3; Biomarker; Apoptosis; Genetic susceptibility; Polymorphism; Head and neck cancer

1. Introduction

Squamous cell carcinoma of the head and neck (SCCHN), including cancers of the oral cavity, pharynx and larynx, is one of the six most common cancers worldwide [1]. In the United States, approximately 53,640 new cases will be diagnosed, resulting in 11,520 deaths in 2013 [2]. Tobacco and alcohol use are the primary risk factors for SCCHN, with the high-risk human papillomavirus (HPV) type 16 as a major risk factor for SCCHN arising in the oropharynx. However, only a fraction of smokers, drinkers and those exposed to HPV will develop SCCHN, suggesting that genetic susceptibility factors modify the risk [3–6]. Increasing evidence has shown that inherited difference in efficiencies of carcinogen metabolism, DNA repair, cell cycle control, apoptosis or a combination of these factors may modulate individual risk of developing SCCHN [7–12].

RASSF proteins, including RASSF1 to RASSF10, are considered potential tumour suppressors, because they play an important role in a myriad of cellular processes, including cell growth, cycle regulation and apoptosis [13–15]. One of the characteristic features of the RASSF family is the Ras-association domain (RA), which can be found either in the C-terminal (RASSF1 to RASSF6) or N-terminal (RASSF7 to RASSF10). The other characteristic feature is the Sav–RASSF–Hpo (SARAH) domain, encoding a protein–protein interaction domain, which is only found in the *RASSF3* gene. The *RASSF3* belongs to a family of RAS effectors and it is the smallest member of the C-terminal RASSF [16]. Unlike other members of the RASSF family, the CpG islands are predicted in the promoter regions of *RASSF3*, but no promoter methylation in *RASSF3* has been reported for human cancers [17–20].

Recent studies have identified that *RASSF3* is a novel tumour suppressor that promotes cell apoptosis [19,20]. For example, it is reported that *RASSF3* expression reduces cell viability and induces apoptosis in human breast cancer cell lines and that MMTV/*RASSF3*-neu bi-transgenic mice displayed a delay in tumour formation [19]; *RASSF3* is also overexpressed in mammary gland of tumour-resistant MMTV/neu mice and significantly upregulated in neu-specific mouse mammary tumours, compared to their adjacent normal tissues; and overexpression of *RASSF3* inhibited cell proliferation in both human and mouse breast cancer cell lines, possibly through induction of apoptosis [19]. More recently, it was found that the tumour suppressor activity of *RASSF3* occurred through p53 stabilisation and regulation of apoptosis and the cell cycle; *RASSF3* expression induced p53-dependent apoptosis, and its depletion attenuated DNA damage-induced apoptosis; *RASSF3* bound to MDM2 and directly interacted with MDM2 and facilitated the ubiquitination of MDM2, thereby increasing p53 stabilisation [20].

RASSF3 is located on chromosome 12 (locus 12q14.2), contains five exons and encodes a 28.6 kDa protein of 238 amino acids [11,16]. Although *RASSF3* is believed to play important roles in the development of multiple cancers, no well-designed and published association studies have assessed the roles of *RASSF3* polymorphisms in cancer risk. Using the bioinformatics tool of single-nucleotide polymorphism (SNP) Function Prediction (FuncPred, <http://snpinfo.niehs.nih.gov/snpfunc.htm>), we identified five common putative

functional SNPs within the 5 kb of the 5' upstream of *RASSF3*, based on the HapMap phase II data in Utah residents with CEU populations [21]. Because of linkage disequilibrium (LD) among these five SNPs, only four were selected and predicted to affect putative transcription factor-binding sites in the *RASSF3* promoter (rs6581580 T>G) or intron 1 region (rs7313765 G>A, rs12311754 G>C and rs1147098 T>C). In the present study, we tested the hypothesis that potential functional SNPs of *RASSF3* are associated with risk of SCCHN in 1087 SCCHN patients and 1090 cancer-free controls of non-Hispanic white population. We further performed laboratory experiments to investigate functional relevance of any of the SNPs that may be associated with SCCHN risk.

2. Materials and methods

2.1. Study subjects

The characteristic details of SCCHN cases and controls used in the present study have been previously reported [22,23]. Briefly, the study population included 1087 non-Hispanic white patients with newly diagnosed, untreated primary tumours of the oral cavity ($n = 319$; 29.3%), oropharynx ($n = 553$; 50.9%) and larynx, and hypopharynx ($n = 215$; 19.8%) seen at the hospital during the period from October 1999 to October 2007. By using frequency matching on age (± 5 years), sex and ethnicity, we identified 1090 cancer-free control subjects from among hospital visitors at the same hospital during the same period. Patients with second SCCHN primary tumours, primary tumours of the nasopharynx or sinonasal tract, or any histopathologic diagnosis other than SCCHN were excluded. Having given a written informed consent, each eligible subject provided additional information about risk factors, such as tobacco smoking and alcohol use, as well as a one-time sample of 30 ml of blood for biomarker tests. Among 1090 cancer-free controls, 102 subjects who had left-over frozen PBMCs (peripheral blood mononuclear cells) with different genotypes for the selected SNPs were included for evaluating mRNA expression levels. The research protocol was approved by the Institutional Review Board.

2.2. Genotyping of *RASSF3*

We extracted genomic DNA from the buffy coat fraction of the blood samples by using a blood DNA mini kit (Qiagen, Inc.) according to the manufacturer's instructions. The DNA purity and concentration were determined by spectrophotometer measurement of absorbance at 260 and 280 nm. Genotyping of the selected SNPs of *RASSF3* (rs6581580 T>G, rs7313765 G>A, rs12311754 G>C and rs1147098 T>C) were performed using the TaqMan methodology in 384-well plates and read with the Sequence Detection Software on an ABI-Prism 7900 instrument according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Primers and probes were supplied by Applied Biosystems. Each plate included four negative controls (no DNA), duplicated positive controls and eight repeat samples. Amplification was done under the following conditions: 50 °C for 2 min, 95 °C for 10 min and 60 °C for 1 min for 40 cycles. In order to test whether or not the polymorphisms of *RASSF3* and *MDM2* interact in their effect on risk of SCCHN, we also genotyped *MDM2* SNP309 (rs2279744) by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method, according to our previous study [24]. For all genotypes of the *RASSF3* gene and *MDM2* SNP309, the assay success rate was >99% and the repeated sample's results were 100% concordant.

2.3. Real-time reverse transcription PCR (RT-PCR) for expression levels of *RASSF3* mRNA in the PBMCs

The expression levels of *RASSF3* mRNA were examined by quantitative RT-PCR using total RNA that was isolated from PBMCs of 102 cancer-free controls with the TRIzol reagent (Invitrogen™, Carlsbad, CA). *RASSF3* mRNA expression levels were detected by

using TaqMan gene expression assays with the master mix reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Each amplification reaction was performed in a final volume of 5 μ l containing 5 ng of the cDNA, 0.25 μ l primers and 2.5 μ l Master mix. Real-time RT-PCR was performed using the ABI-Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). The 5 μ l reaction mixtures were incubated in a 384-well optical plate at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Each sample was analysed in duplicate and expression levels of *RASSF3* mRNA were calculated relative to that of *18S*.

2.4. Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted from human head and neck carcinoma cell line UMSCC-17B, as previously described [25]. Complementary single-stranded oligonucleotides for rs6581580 of *RASSF3* (5'-AGCACGGCTGACCGATACCAAGCAGAGAA CC-3' for the T allele and 5'-AGCACGGCTGACCGAGACCAAGCAGAGAACC- 3' for the G allele) were biotin-labelled using the 3'-end biotin labelling kit (Thermo Scientific, Rockford, IL) and re-annealed to double strand, and identical but unlabelled oligonucleotides with the same sequence were used as competitors.

The EMSA assay was performed using the Light Shift Chemiluminescent EMSA kit (Thermo Scientific, Rockford, IL) according to the experimental procedures provided by the manufacturer. Briefly, 10 μ g of nuclear protein extracts were incubated with 3' biotin-labelled DNA oligonucleotides at room temperature for 30 min. The specificity of *RASSF3* and DNA binding activity was determined by adding a 50-fold excess of unlabelled oligonucleotides for competition reactions. The DNA-protein complex was separated on 6% polyacrylamide gel, and the products were detected by Stabilised Streptavidin-Horseradish Peroxidase Conjugate (Thermo Scientific, Rockford, IL).

2.5. Statistical analysis

We evaluated differences in select demographic variables, risk factors and frequencies of the *RASSF3* genotypes between cases and controls by using the χ^2 test and examined Hardy-Weinberg equilibrium by a goodness-of-fit χ^2 test to compare the observed genotype frequencies with the expected ones among the controls. We estimated associations between *RASSF3* variants/ genotypes and SCCHN risk by computing odds ratios (ORs) and 95% confidence intervals (CIs) from both univariate and multivariate logistic regression analyses with adjustment for the known risk factors for SCCHN, such as age, sex, smoking and alcohol status. Subjects who had smoked <100 cigarettes in their lifetime were defined as never smokers; all others were defined as ever smokers. Among ever smokers, those who had quit and had not smoked for >1 year were defined as former smokers, and the others were defined as current smokers. Similarly, 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 and the others as current drinkers. Multiplicative interactions of SNP-pairs between *RASSF3* and *MDM2* were tested using unconditional logistic regression with the inclusion of the main effects of SNPs, interaction terms and other risk factors. We also used the false-positive report probability (FPRP) to test for false positive associations [26]. For all significant genetic effects observed in our study, we calculated FPRP with prior probabilities of 0.0001, 0.001, 0.01, 0.1 and 0.25. The OR was set close to the observed value in our study, and a probability <0.2 was considered a noteworthy. All tests were two sided and $P < 0.05$ was considered significant. All statistical

analyses were performed with SAS software (version 9.1.3; SAS Institute, Inc., Cary, NC), unless stated otherwise.

3. Results

3.1. Characteristics of study subjects

The distributions of selected characteristics of the cases and controls are presented in Table 1. There were no significant differences in the distributions of age and sex between 1087 cases and 1090 controls with similar age distribution ($P = 0.536$) with mean ages of 57.1 (± 11.1) and 56.6 (± 11.0) years, respectively. There were more current smokers (37.8%) and current drinkers (50.9%) in the cases than in the controls (14.5% and 40.5%, respectively; $P < 0.001$ for both). However, all these variables were further adjusted for any residual confounding effect in later multivariate logistic regression analysis.

3.2. Overall associations between RASSF3 variants/ genotypes and risk of SCCHN

The genotype and allele frequencies of the selected SNPs and their associations with risk of SCCHN are summarised in Table 2. The genotype distributions of these SNPs in the controls were in agreement with the Hardy–Weinberg equilibrium ($P = 0.672$ for rs6581580, 0.505 for rs12311754 and 0.400 for rs1147098), except for rs7313765 ($P = 0.024$). Compared with the rs6581580 TT genotype, the variant TG, GG and GG/TG genotypes were associated with a statistically significantly reduced risk of SCCHN (adjusted OR = 0.75; 95% CI, 0.62–0.92; $P = 0.006$ for TG, adjusted OR = 0.74; 95% CI = 0.58–0.95; $P = 0.021$ for GG and adjusted OR = 0.75; 95% CI = 0.62–0.91; $P = 0.003$ for GG/TG; $P_{\text{trend}} = 0.012$). For the rs7313765, the variant GA and AA/GA genotypes were also associated with a statistically significantly reduced risk of SCCHN (adjusted OR = 0.70; 95% CI = 0.57–0.85; $P = 0.0004$ for GA, adjusted OR = 0.73; 95% CI = 0.60–0.88; $P = 0.001$ for AA/GA; $P_{\text{trend}} = 0.041$), compared with the GG genotype. However, no associations were observed between the genotypes of other two *RASSF3* SNPs (rs12311754 G>C and rs1147098 T>C) and *MDM2* SNP309 (rs2279744) and SCCHN risk (Table 2).

The LD analysis showed that all four SNPs were not in LD in the controls ($D' = 0.83$ and $r^2 = 0.65$ for rs6581580 T>G and rs7313765 G>A; $D' = 0.50$ and $r^2 = 0.04$ for rs6581580 T>G and rs12311754 G>C; $D' = 0.77$ and $r^2 = 0.42$ for rs6581580 T>G and rs1147098 T>C; $D' = 0.84$ and $r^2 = 0.12$ for rs7313765 G>A and rs12311754 G>C; $D' = 0.99$ and $r^2 = 0.66$ for rs7313765 G>A and rs1147098 T>C; $D' = 0.87$ and $r^2 = 0.19$ for rs12311754 G>C and rs1147098 T>C; Fig. 1A). These suggest that each SNP may have an independent effect. Taking into account possible joint effects from different variants and potential locu–locu interactions of the *RASSF3* SNPs on risk of SCCHN, we then examined the combined effects of these four variants by the number of putative risk alleles (i.e. rs6581580T, rs7313765G, rs12311754C and rs1147098C). As shown in Table 2, when we used ‘0–4’ risk alleles as the reference, we found that the ‘5–8’ risk allele group was significantly associated with risk of SCCHN (adjusted OR = 1.37, 95% CI = 1.13–1.66; $P_{\text{trend}} = 0.001$).

3.3. Associations between RASSF3 variants and risk of SCCHN by tumour sites

To investigate tumour-site specific effects of *RASSF3* variants on risk of SCCHN, we conducted the subgroup analysis separately for oropharyngeal cancers and nonoropharyngeal cancers (including oral cavity, hypopharynx or larynx). As shown in Table 2, rs6581580 variant genotypes were associated with a significantly decreased risk of SCCHN only for non-oropharynx sites (TG versus TT: adjusted OR = 0.70, 95% CI = 0.54–0.91; GG versus TT: adjusted OR = 0.61, 95% CI = 0.44–0.85; GG/TG versus TT: adjusted OR = 0.68, 95% CI = 0.53–0.86, $P_{\text{trend}} = 0.048$). Likewise, rs7313765 variant genotypes were also associated with a significantly decreased risk of SCCHN only for non-oropharynx

sites (GA versus GG: adjusted OR = 0.58, 95% CI = 0.45–0.75; AA versus GG: adjusted OR = 0.67, 95% CI = 0.48–0.95; AA/GA versus GG: adjusted OR = 0.60, 95% CI = 0.47–0.77). Consistent the overall analysis, no association with risk of SCCHN was observed for rs12311754 and rs1147098 variant genotypes in both subgroups. In addition, the locus-dose effect of combined risk alleles was also seen in non-oro-pharyngeal cancers ($P_{\text{trend}} < 0.001$). In the dichotomised groups, the group with ‘5–8’ risk alleles had a significantly higher risk of non-oro-pharynx sites (adjusted OR = 1.63, 95% CI = 1.27–2.08), compared with ‘0–4’ group. However, no significant associations were observed for the oro-pharynx site (adjusted OR = 1.21, 95% CI = 0.96–1.52; $P = 0.105$).

3.4. Stratification analyses

We then performed stratified analyses to evaluate the effects of variant genotypes on the risk of SCCHN by age, sex, smoking status, alcohol use and stage (Table 3). The results showed that the decreased risk associated with the variant genotypes of rs6581580 and rs7313765 was more evident in the older subjects (adjusted OR = 0.69 and 0.62, 95% CI = 0.52–0.92 and 0.47–0.83, respectively), females (adjusted OR = 0.53 and 0.53, 95% CI = 0.35–0.79 and 0.36–0.80, respectively), ever smokers (adjusted OR = 0.75 and 0.68, 95% CI = 0.59–0.96 and 0.54–0.87, respectively), never drinkers (adjusted OR = 0.69, 95% CI = 0.50–0.92 for 6581580) and ever drinkers (adjusted OR = 0.75, 95% CI = 0.60–0.95 for 7313765). Additionally, the increased risk associated with the combined risk alleles was also more pronounced among the older subjects (adjusted OR = 1.18, 95% CI = 1.15–2.02), females (adjusted OR = 1.97, 95% CI = 1.32–2.95), ever smokers (adjusted OR = 1.39, 95% CI = 1.09–1.77), never and ever drinkers (adjusted OR = 1.40 and 1.28, 95% CI = 1.02–1.91 and 1.01–1.61) (Table 3).

We also explored the interaction between the two *RASSF3* (rs6581580 and rs7313765) SNP and *MDM2* SNP309, but the associated with these two SNPs and their combined risk alleles was not different by *MDM2* SNP309 (Table 3), indicating that there was no interaction between them ($P_{\text{interaction}} > 0.05$) (Supplementary Table 1).

Because most of the significant findings were in the subgroup analysis, we calculated the FPRP values for all the observed significant associations. As shown in (Table 4), when the assumption of prior probability was 0.25, the association with *RASSF3* (rs6581580 GG/TG and rs7313765 AA/GA) genotypes and was still noteworthy for all subjects (FPRP = 0.011 and 0.003, respectively), non-oro-pharynx (FPRP = 0.004 and <0.001, respectively), age >57 years (FPRP = 0.034 and 0.004, respectively), females (FPRP = 0.009 and 0.012, respectively), as well as for never drinkers (FPRP = 0.034 for rs6581580) and ever smokers and ever drinkers (FPRP = 0.006 and 0.049 for rs7313765, respectively). When the assumption of prior probability was 0.1, the association with *RASSF3* (rs6581580 GG/ TG and rs7313765 AA/GA) genotypes and was still noteworthy for all subjects (FPRP = 0.031 and 0.009, respectively), non-oro-pharynx (FPRP = 0.012 and 0.001, respectively), age >57 years (FPRP = 0.013 for rs7313765), females (FPRP = 0.0026 and 0.036, respectively) and ever smokers (FPRP = 0.019 for rs7313765).

3.5. Function assay for the SNP in the promoter region of *RASSF3*

To further characterise biological significance of the *RASSF3* rs6581580, we conducted correlation analysis between rs6581580 and rs7313765 genotypes and expression levels of *RASSF3* mRNA in PBMCs samples from 102 cancer-free controls. We found that, in the 102 cancer-free controls, 29 had the rs6581580 TT genotype, 55 had the TG genotype and 18 had the GG genotype, the genotype distribution of the SNP was in agreement with the Hardy–Weinberg equilibrium ($P = 0.357$). As shown in Fig. 1B, the *RASSF3* mRNA relative expression was higher for the GG genotype (16.62 ± 0.74) than the TT genotype ($16.11 \pm$

0.82), and this difference was statistically significant ($P = 0.038$). Although the *RASSF3* mRNA relative expression for the TG genotype (16.38 ± 0.79) and the GG/TG genotypes (16.44 ± 0.78) were not significantly different from that for the TT genotype ($P = 0.140$ and $P = 0.059$, respectively), the trend test for the effect of the G allele on the expression was towards significance ($P_{\text{trend}} = 0.029$) (Fig. 1B). In the 102 cancer-free controls, 23 had the rs7313765 GG genotype, 59 had the GA genotype, and 20 had the AA genotype, the genotype distribution of the SNP was in agreement with the Hardy–Weinberg equilibrium ($P = 0.111$). However, as shown in Fig. 1C, the relative *RASSF3* mRNA expression levels for the rs7313765 AA, GA and AA/GA genotypes were not significantly different from that for the GG genotype ($P = 0.696$, 0.335 and 0.378 , respectively). Because rs12311754 G>C, and rs1147098 T>C genotypes were not consistently associated with SCCHN risk, their correlation with the related mRNA expression levels were not pursued in further laboratory studies.

We then further examined whether nuclear proteins, including transcriptional factors, could bind to this sequence by using EMSA. As shown in Fig. 1D, the probe of the *RASSF3* rs6581580 T or G allele was able to bind to a nuclear protein; however, the binding ability of the probe of the variant G allele to the protein had a significantly stronger binding affinity to the nuclear protein extracts than that for the T allele (Fig. 1D, lanes 4 versus 1). Competition assays further showed that the formation of the DNA–protein complexes was completely eliminated by the 50-fold excess of unlabelled probes containing either T or G alleles (Fig. 1D, lanes 2, 3 and lanes 5, 6), indicating that the binding was sequence-specific. Because there were no significant correlation between the relative expression levels of *RASSF3* mRNA and rs7313765 genotypes, its functional assay was not pursued in further laboratory studies.

4. Discussion

In this hospital-based case–control study of 1087 patients with SCCHN and 1090 cancer-free controls in a non-Hispanic white population, we evaluated the associations between four common functional SNPs of the *RASSF3* gene and risk of SCCHN. We found that both variant rs6581580G allele (GG/GT) and rs7313765A allele (AA/GA) were associated with a significantly reduced risk of SCCHN. Stratified analyses showed that the protective effect was more evident in subgroups of subjects >57 years, females, ever smokers, and patients with non-opharyngeal cancer. Although multiple testings have been performed in the study, the results of FPRP indicated that our results were less likely to be false positives. These findings are consistent with the notion that *RASSF3* SNPs may be involved in cancer risk associated with exposure to tobacco smoke, because old subjects and ever smokers are likely to have a higher level of exposure to smoke, of whom females are more susceptible, and non-opharyngeal cancer are known to be tobacco smoke related, compared with oropharyngeal cancer that is unlikely to be tobacco smoke related but more to be HPV-infection related [3,5,6,27].

The combined genotypes of four functional *RASSF3* SNPs were also significantly associated with risk of SCCHN, although rs6581580G and rs7313765A alleles were more likely to have contributed to the observed cancer risk. Additional functional experiments consistently demonstrated that the *RASSF3* SNP rs6581580 T-to-G allelic change contributed to the increased promoter activity and that the mechanism of specific effects of the rs6581580 T>G SNP may be due to its modification of nuclear protein binding affinity. Collectively, these data suggest that functional *RASSF3* SNPs may modify risk of SCCHN; in particular, it is likely that the *RASSF3* (rs6581580) polymorphism may mediate expression levels of *RASSF3* and thus play a role in the aetiology SCCHN.

Although neither transcriptional regulation nor epigenetic modification of *RASSF3* expression has been fully understood, *RASSF3*, as a RAS effector or a tumour suppressor gene, is believed to play important roles in the development of multiple cancers; it appears that *RASSF3* expression is tumour-type specific in humans [19]. For example, it has been demonstrated that expression of *RASSF3* is upregulated in breast and cervix tumours but down regulated in tumours of human lung, uterus, stomach, colon and liver [19]. Other studies have demonstrated that promoter and some intronic SNPs may alter the mRNA levels by affecting transcription and RNA elongation, splicing or maturation [28–30]. In the current study, the results showed that the variant rs6581580G allele affected the *RASSF3* transcription, and further EMSA analysis suggested that a higher promoter activity of the sequences containing the variant G allele may be regulated by the direct DNA–protein binding. Furthermore, we also found that variant genotypes of rs6581580 were associated with increased mRNA expression levels of *RASSF3* in PBMCs from cancer-free controls, suggesting a potentially functional impact of this promoter SNP on the mRNA levels, which thus supports a role in the susceptibility to SCCHN. In contrast, we did not observe such a genotype–phenotype correlation for the other *RASSF3* intronic (rs7313765) SNP.

Although the mechanisms through which *RASSF3* functions as a tumour suppressor remain unknown, it is reported that *RASSF3* plays a role in p53-dependant apoptosis through *RASSF3* binding to MDM2 and thus directly interacts with MDM2 and facilitates the ubiquitination of MDM2, thereby increasing p53 stabilisation [20]. Because MDM2 is a key negative regulator of the p53 activity, overexpression of MDM2 results in the inhibition of p53-mediated-transcriptional activation, thereby contributing to human carcinogenesis [31]. It had been demonstrated that 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 tumour suppressor activity and potentially cancer development in humans [32,33]. To evaluate the joint effect of polymorphisms of *RASSF3* and *MDM2* in risk of SCCHN, we explored the interactive effect of the two *RASSF3* SNPs (rs6581580 and rs7313765) and *MDM2* SNP309, but we did not observe any statistical evidence for the perceived interaction between them. Consistent with this result, our mini meta-analysis using the published data and our previously study both showed that *MDM2* SNP309 was not significantly associated with risk of SCCHN [24], although several studies observed that *MDM2* SNP309T>G was associated with risk of head and neck cancer, which had relatively small sample size. It is also possible that the effect of the *MDM2* SNP309 on risk of SCCHN may be modest and thus could not be detected in the present study, or the effect may be modified by untyped SNPs of other genes.

To our knowledge, this is the first study to evaluate the associations between polymorphisms in the *RASSF3* motif and risk of SCCHN. However, the present study also had several limitations. Firstly, we were unable to explore the exact mechanisms by which *RASSF3* SNPs influence SCCHN risk. Secondly, it is a hospital-based, case–control study, and inherent selection bias cannot be completely excluded. However, the agreement of observed genotype distributions with Hardy–Weinberg equilibrium and the similar allele frequencies of our controls to those reported in HapMap – CEU populations from the dbSNP database (i.e. 0.457 versus 0.505 for rs6581580 G allele, 0.444 versus 0.451 for rs7313765 A allele, 0.177 versus 0.161 for rs12311754 C allele, and 0.458 versus 0.437 for rs1147098, respectively) suggested that selection bias in terms of genotype distribution should not be substantial, if any. Third, although our study had over 1000 SCCHN cases and 1000 controls, our sample size was still not large enough to identify weak associations in some subgroups and the anticipated gene–environment interactions. Finally, we used the common functional SNPs in our investigation, which did not include all representative SNPs in the entire gene. Some other rare functional SNPs, which may influence risk of SCCHN, may

have been missed. These issues need to be further addressed and validated in studies with different cancer sites and large sample sizes, preferably including different ethnic groups.

In conclusion, in this hospital-based case-control study, we found that two potentially functional SNPs (rs6581580 T>G and rs7313765 G>A) were significantly associated with reduced risk of SCCHN in a non-Hispanic white population, especially those cancers arising at non-oro-pharyngeal sites. Our study suggested that the *RASSF3* promoter rs6581580 T>G polymorphism is functional, modulating susceptibility to SCCHN by altering the gene expression levels. We also found that all four potentially functional SNPs of *RASSF3* may have a joint effect on the risk of SCCHN, especially for non-oro-pharyngeal cancers. These results indicate the important role of the *RASSF3* variants in SCCHN carcinogenesis, but our findings need to be validated by larger studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejca.2013.11.009>.

Fig. 1.

(A) Positions of the four potentially functional single-nucleotide polymorphisms (SNPs) in *RASSF3* and pairwise linkage disequilibrium (LD) between them. These four SNPs were predicted to affect putative transcription factor-binding activity. (B) Genotype–phenotype correlation for rs6581580 and relative expression levels of *RASSF3* mRNA in peripheral blood mononuclear cells (PBMCs) of 102 cancer-free controls. The relative *RASSF3* mRNA expression levels were higher for rs6581580 GG genotype (16.62 ± 0.74) than that for the TT genotype (16.11 ± 0.82), and the difference was statistically significant ($P < 0.038$), the TG genotype (16.38 ± 0.79) and the GG/TG genotypes (16.44 ± 0.78) were not significantly different from that for the TT genotype ($P = 0.140$, and $P = 0.059$, respectively). (C) Genotype–phenotype correlation for rs7313765 and relative expression levels of *RASSF3*

mRNA in PBMCs of 102 cancer-free controls. The relative *RASSF3* mRNA expression levels were similar among the three groups with rs7313765 GG, GA and AA genotypes ($P = 0.696$ for GG versus AA, $P = 0.335$ for GG versus GA and $P = 0.378$ for GG versus AA/GA). (D) Electrophoretic mobility shift assay (EMSA) for comparison of DNA–protein binding activities between labelled probes containing different alleles of rs6581580. As it shown in lane 4 and lane 1, probe containing G allele had a stronger binding affinity to the nuclear protein extracts than probe containing T allele. Competition assays further showed that the formation of the DNA–protein complexes was completely eliminated by the 50-fold excess of unlabelled probes containing either T or G alleles (lanes 2, 3 and lanes 5, 6), which indicated that the binding was sequence-specific.

Frequency distributions of selected variables in squamous cell carcinoma of the head and neck (SCCHN) cases and cancer-free controls.

Table 1

Variables	Cases (<i>n</i> = 1087)		Controls (<i>n</i> = 1090)		<i>p</i> ^{<i>a</i>}
	<i>n</i>	%	<i>n</i>	%	
Age (years)					0.536
50	299	27.5	312	28.6	
51–57	281	25.9	260	23.9	
>57	507	46.6	518	47.5	
Sex					0.525
Female	270	24.8	258	23.7	
Male	817	75.2	832	76.3	
Smoking status					<0.001
Never	304	28.0	535	49.1	
Former	372	34.2	397	36.4	
Current	411	37.8	158	14.5	
Alcohol use					<0.001
Never	296	27.2	473	43.4	
Former	238	21.9	176	16.2	
Current	553	50.9	441	40.5	
Tumour site					
Oropharynx	553	50.9			
Non-oropharynx ^{<i>b</i>}	534	49.1			
Stage					
I	116	10.6			
II	154	14.2			
III	186	17.1			
IV	631	58.1			

^{*a*}Two-sided χ^2 test.
^{*b*}Included oral cavity (*n* = 319), larynx (*n* = 172) and hypopharyngeal (*n* = 43) cancer cases.

Table 2

Association between *RASSF3* genotypes and squamous cell carcinoma of the head and neck (SCCHN) risk.

Genotypes	Controls (%)		Overall		Oropharynx		Non-oropharynx	
	Cases (%)	Odds ratio (OR) (95% confidence interval (CI)) ^a	Cases (%)	OR (95% CI) ^a	Cases (%)	OR (95% CI) ^a	Cases (%)	OR (95% CI) ^a
All subjects	1090 (100.0)		1087 (100.0)		553 (100.0)		534 (100.0)	
<i>rs6581580</i> : T>G	1090		1086		553		533	
TT	325 (29.8)	1.00	379 (34.9)	1.00	189 (34.2)	1.00	190 (35.6)	1.00
TG	534 (49.0)	0.75 (0.62–0.92)	500 (46.0)	0.75 (0.62–0.92)	249 (45.0)	0.79 (0.62–1.01)	251 (47.1)	0.70 (0.54–0.91)
GG	231 (21.2)	0.74 (0.58–0.95)	207 (19.1)	0.74 (0.58–0.95)	115 (20.8)	0.86 (0.64–1.15)	92 (17.3)	0.61 (0.44–0.85)
<i>P</i> _{trend}		0.012				0.168		0.048
GG/TG	765 (70.2)	0.75 (0.62–0.91)	707 (65.1)	0.75 (0.62–0.91)	364 (65.8)	0.81 (0.65–1.02)	343 (64.4)	0.68 (0.53–0.86)
<i>rs12311754</i> : G>C	1089		1084		552		532	
GG	734 (67.4)	1.00	730 (67.3)	1.00	378 (68.5)	1.00	352 (66.2)	1.00
GC	324 (29.8)	0.98 (0.81–1.19)	316 (29.2)	0.98 (0.81–1.19)	156 (28.3)	0.95 (0.75–1.20)	160 (30.0)	1.04 (0.81–1.33)
CC	31 (2.8)	1.37 (0.83–2.26)	38 (3.5)	1.37 (0.83–2.26)	18 (3.2)	1.20 (0.65–2.20)	20 (3.8)	1.85 (0.99–3.46)
<i>P</i> _{trend}		0.444				0.754		0.153
CC/GC	355 (32.6)	1.02 (0.84–1.23)	354 (32.7)	1.02 (0.84–1.23)	174 (31.5)	0.97 (0.78–1.22)	180 (33.8)	1.10 (0.86–1.40)
<i>rs1147098</i> : T>C	1090		1087		553		534	
TT	313 (28.7)	1.00	313 (28.8)	1.00	167 (30.2)	1.00	146 (27.3)	1.00
TC	555 (50.9)	0.94 (0.76–1.15)	528 (48.6)	0.94 (0.76–1.15)	268 (48.5)	0.90 (0.70–1.15)	260 (48.7)	1.02 (0.78–1.34)
CC	222 (20.4)	1.14 (0.89–1.47)	246 (22.6)	1.14 (0.89–1.47)	118 (21.3)	0.98 (0.72–1.32)	128 (24.0)	1.44 (1.04–2.00)
<i>P</i> _{trend}		0.230				0.669		0.041
CC/TC	777 (71.3)	1.00 (0.82–1.21)	774 (71.2)	1.00 (0.82–1.21)	386 (69.8)	0.92 (0.73–1.16)	388 (72.7)	1.14 (0.88–1.47)
<i>rs7313765</i> : G>A	1088		1083		551		532	
GG	318 (29.2)	1.00	383 (35.4)	1.00	182 (33.0)	1.00	201 (37.8)	1.00
GA	574 (52.8)	0.70 (0.57–0.85)	506 (46.7)	0.70 (0.57–0.85)	263 (47.7)	0.80 (0.63–1.02)	243 (45.7)	0.58 (0.45–0.75)
AA	196 (18.0)	0.83 (0.64–1.08)	194 (17.9)	0.83 (0.64–1.08)	106 (19.3)	0.96 (0.71–1.31)	88 (16.5)	0.67 (0.48–0.95)
<i>P</i> _{trend}		0.041				0.164		0.003
AA/GA	770 (70.8)	0.73 (0.60–0.88)	700 (64.6)	0.73 (0.60–0.88)	369 (67.0)	0.84 (0.67–1.06)	331 (62.2)	0.60 (0.47–0.77)
Combined effect of risk alleles in <i>RASSF3</i>	1090 (100.0)		1086 (100.0)		553		533	
Dichotomy								

Genotypes	Controls (%)		Overall	Oropharynx		Non-oropharynx	
		Cases (%)	Odds ratio (OR) (95% confidence interval (CI)) ^a	Cases (%)	OR (95% CI) ^a	Cases (%)	OR (95% CI) ^a
0-4	770 (70.8)	703 (65.1)	1.00	369 (67.1)	1.00	334 (63.0)	1.00
5-8	317 (29.2)	377 (34.9)	1.37 (1.13-1.66)	181 (32.9)	1.21 (0.96-1.52)	196 (37.0)	1.63 (1.27-2.08)
<i>P</i> _{trend}			0.001		0.1054		<0.0001
MDM 309T>G (rs2279744)	1088	1080		549		531	
TT	486 (44.7)	462 (42.8)	1.00	237 (43.2)	1.00	225 (42.4)	1.00
TG	472 (43.4)	486 (45.0)	1.11 (0.92-1.34)	247 (45.0)	1.09 (0.87-1.37)	239 (45.0)	1.15 (0.90-1.46)
GG	130 (11.9)	132 (12.2)	1.05 (0.79-1.40)	65 (11.8)	1.03 (0.73-1.46)	67 (12.6)	1.08 (0.75-1.57)
<i>P</i> _{trend}			0.464		0.632		0.428
GG/TG	602 (55.3)	618 (57.2)	1.10 (0.92-1.31)	312 (56.8)	1.08 (0.87-1.34)	306 (57.6)	1.13 (0.90-1.43)

^a Adjusted for age, sex, smoking status and alcohol use in a logistic regression model.

Stratification analysis for associations between *RASSF3* variant genotypes and risk of squamous cell carcinoma of the head and neck (SCCHN).

Table 3

Variables	rs7313765 (case/control)				Adjusted OR ^a (95% CI)		Combined effect of risk alleles ^b		Adjusted OR ^a (95% CI)
	Adjusted odds ratio (OR) ^a (95% confidence interval (CI))				GG	AA/GA	0-4	5-8	
	rs6581580 (case/control)	GG/TG	TT						
Age, year									
57 (median)	206/176	373/396	0.79 (0.61-1.02)	206/178	372/393	0.82 (0.63-1.06)	373/401	203/170	1.29 (1.00-1.67)
>57 (median)	173/149	334/369	0.69 (0.52-0.92)	177/140	328/377	0.62 (0.47-0.83)	330/369	174/147	1.18 (1.15-2.02)
Gender									
Females	97/68	173/190	0.53 (0.35-0.79)	98/65	170/193	0.53 (0.36-0.80)	168/193	100/65	1.97 (1.32-2.95)
Males	282/257	534/575	0.83 (0.67-1.03)	285/253	530/577	0.80 (0.65-1.00)	535/577	277/252	1.22 (0.99-1.52)
Smoking status									
Never	113/172	190/363	0.77 (0.57-1.04)	105/168	197/367	0.85 (0.63-1.14)	196/368	106/166	1.23 (0.91-1.67)
Ever	266/153	517/402	0.75 (0.59-0.96)	278/150	502/403	0.68 (0.54-0.87)	507/402	271/151	1.39 (1.09-1.77)
Alcohol status									
Never	114/145	181/328	0.69 (0.50-0.92)	106/141	189/331	0.74 (0.54-1.01)	187/331	108/141	1.40 (1.02-1.91)
Ever	265/180	526/437	0.83 (0.65-1.05)	277/177	510/439	0.75 (0.60-0.95)	516/439	269/176	1.28 (1.01-1.61)
MDM2 T309G									
TT	163/140	299/346	0.73 (0.55-0.98)	158/134	304/352	0.73 (0.55-0.98)	306/348	156/138	1.32 (0.99-1.77)
TG + GG	215/185	403/417	0.77 (0.60-0.99)	225/184	393/418	0.73 (0.56-0.94)	395/422	221/179	1.41 (1.09-1.82)
Stage									
I-II	97	172	1.00	96	173	1.00	173	95	1.00
III-IV	282	535	0.93 (0.70-1.25)	287	526	0.99 (0.74-1.33)	530	282	1.02 (0.76-1.37)

^a Adjusted for age, sex, smoking status and alcohol status (the stratified factor in each stratum excluded).

^b The number represents the numbers of unfavored alleles for each single-nucleotide polymorphism (SNP).

False-positive report probability (FPRP) values for associations between risk of squamous cell carcinoma of the head and neck (SCCHN) and frequencies of rs6581580 and rs7313765Genotypes.

Table 4

Genotype	Positive odds ratio (OR) 95% confidence interval (CI) ^a	p ^b	Statistical power ^c	Prior probability				
				0.25	0.1	0.01	0.001	0.0001
rs6581580: T>G	TT versus GG/TG							
All subjects	0.75 (0.62–0.91)	0.004	1.000	0.011	0.031	0.260	0.780	0.973
Non-oro-pharynx	0.68 (0.53–0.86)	0.001	0.995	0.004	0.012	0.114	0.564	0.928
Age >57	0.69 (0.52–0.92)	0.011	0.986	0.034	0.095	0.535	0.921	0.991
Females	0.53 (0.35–0.79)	0.002	0.613	0.009	0.026	0.228	0.748	0.968
Ever smokers	0.75 (0.59–0.96)	0.022	0.999	0.063	0.168	0.689	0.957	0.996
Never drinkers	0.69 (0.50–0.92)	0.011	0.986	0.034	0.096	0.535	0.921	0.991
rs7313765: G>A	GG versus AA/GA							
All subjects	0.73 (0.60–0.88)	<0.001	1.000	0.003	0.009	0.087	0.491	0.906
Non-oro-pharynx	0.60 (0.47–0.77)	<0.001	0.924	<0.001	0.001	0.006	0.061	0.393
Age >57	0.62 (0.47–0.83)	0.001	0.926	0.004	0.013	0.124	0.587	0.934
Females	0.53 (0.36–0.80)	0.003	0.609	0.012	0.036	0.290	0.804	0.976
Ever smokers	0.68 (0.54–0.87)	0.002	0.993	0.006	0.019	0.077	0.685	0.956
Ever drinkers	0.75 (0.60–0.95)	0.017	1.000	0.049	0.133	0.628	0.945	0.994

^aThe adjusted OR.

^bThe omnibus chi-square test of the genotype frequency distributions.

^cCalculated using study subjects to detect an OR of 2.00 with the common genotype used as the reference.