A functional variant at the *miR-184* binding site in *TNFAIP2* and risk of squamous cell carcinoma of the head and neck

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Although the role of *TNFAIP2* is still unclear, it is an important gene involved in apoptosis, and there are single-nucleotide polymorphisms (SNPs) at its microRNA (miRNA)-binding sites that could modulate miRNA target gene function. In this study, we evaluated associations of four selected SNPs (rs8126 T > C, rs710100 G > A, rs1052912 G > A and rs1052823 G > T) in the miRNA-binding sites of the 3' untranslated region (UTR) with squamous cell carcinoma of the head and neck (SCCHN) risk in 1077 patients with SCCHN and 1073 cancer-free controls in a non-Hispanic White population. We found that, compared with the rs8126 TT genotype, the variant C allele were associated with increased SCCHN risk in an allele dose-response manner (adjusted odds ratio = 1.48 and 95% confidence interval = 1.06–2.05 for CC, respectively; $P_{\text{trend}} = 0.009$). No significant associations were seen for the other three SNPs (rs710100 G > A, rs1052912 G > A and rs1052823 G > T). Additionally, we identified that the rs8126 T > C SNP is within the *miR-184* seed binding region in the 3' UTR of *TNFAIP2*. Further functional analyses showed that the rs8126 variant C allele led to significantly lower luciferase activity, compared with the T allele. In the genotype-phenotype correlation analysis of peripheral blood mononuclear cells from 64 SCCHN patients, the rs8126 CC genotype was associated with reduced expression of *TNFAIP2* messenger RNA. Taken together, these findings indicate that the *miR-184* binding site SNP (rs8126 T > C) in the 3' UTR of *TNFAIP2* is functional by modulating *TNFAIP2* expression and contributes to SCCHN susceptibility. Larger replication studies are needed to confirm our findings.

Introduction

Squamous cell carcinoma of the head and neck (SCCHN), which includes cancers of the oral cavity, pharynx and larynx, is one of the six most common cancers worldwide (1). In the USA, ~49 260 new cases were diagnosed, and 10 480 deaths occurred in 2010 (2). Although tobacco and alcohol use play a role in the etiology of SCCHN, high-risk human papillomavirus types 16 and 18 can also play a role in the pathogenesis of SCCHN, particularly for tumors that arise in the oropharynx. The fact that only a fraction of smokers, drinkers and people exposed to human papillomavirus develop SCCHN suggests that additional environmental and genetic factors also contribute to the disease (3). Indeed, tobacco carcinogens cause a variety of types of DNA damage in the target cells. To avoid uncontrolled cell growth due to mutations resulting from DNA damage, these cells must initiate cell cycle control mechanisms that repair

Abbreviations: CI, confidence interval; FPRP, false-positive report probability; mRNA, messenger RNA; miRNA, microRNA; NPC, nasopharyngeal carcinoma; OR, odds ratio; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cell; RT, reverse transcription; SCCHN, squamous cell carcinoma of the head and neck; SNP, single-nucleotide polymorphism; TNFAIP, tumor necrosis factor- α -induced protein; UTR, untranslated region.

DNA damage or initiate apoptosis to eliminate cells with overwhelming damage.

Recent studies have indicated that microRNAs (miRNAs) play an important role in cell differentiation, proliferation, cell cycle progression and apoptosis (4,5). miRNAs are single-stranded 21–23 nucleotide long RNA molecules encoded in the genome (6,7). miRNAs can negatively regulate target gene transcription through hybridization to incomplete complementary sequences in the 3' untranslated region (UTR) of their target messenger RNAs (mRNAs). This process results in either the degradation of target mRNAs or repression of their translation. Elevated or decreased expression of miRNAs has been found in tumors of various types, and recent studies have demonstrated that miRNAs may function as tumor suppressors and/or oncogenes in many cancers, including SCCHN (8–11).

The role of miRNAs' genetic variants in cancer susceptibility is an emerging research interest because sequence variations in miRNA-binding sites and target genes, such as single-nucleotide polymorphisms (SNPs), may modulate miRNA expression and thus the expression (mRNA) levels of the target genes. Therefore, it is important to understand the functional and evolutionary significance of genetic variants in microRNAs and mRNAs that interact with each other as well as with environmental risk factors in the associated biological end points (12–15). For example, it has been shown that SNPs located in miRNA-binding sites (miRNA-binding SNPs) may affect miRNA expression and function of the target genes and thus may potentially be involved in carcinogenesis (16,17).

Although some studies have investigated miRNA expression in SCCHN tissues and cancer cell lines (18,19), few studies have investigated genetic variants and levels of expression of miRNAs and target genes as a risk factor for SCCHN. Our previous studies suggest that genetic variants in some apoptotic genes are associated with risk of SCCHN, and miRNAs are known to regulate apoptotic genes (20–22). The tumor necrosis factor- α -induced proteins (TNFAIPs), including TNFAIP1 to TNFAIP8, are pro-apoptotic proteins (23); TNFAIP2 (also called primary response gene B94 protein) belongs to the SEC6 family and is differentially expressed in the development and capillary tube-like formation *in vitro*. *TNFAIP2* was originally described as a novel tumor necrosis factor- α -induced gene in human endothelial cells (24). Although the function of the TNFAIP2 protein is unknown, it has been identified as an apoptosis-related gene targeted by retinoic acid in acute pro-myelocytic leukemia and other cancers, and *TNFAIP2* mRNA is detectable in many human tissues and most hematopoietic cell lines (25–27). More recently, the *TNFAIP2* mRNA and protein were found to be highly expressed in nasopharyngeal carcinoma (NPC) tumor cells compared with adjacent normal tissues; the increased expression of *TNFAIP2* was significantly associated with shorter distant metastasis-free survival in NPC patients (28). More importantly, this gene has SNPs in the miRNA-binding sites that allow us to demonstrate their biological effects related to miRNA.

The *TNFAIP2* gene is located on chromosome 14q32 and encodes 654 amino acids of a 73 kDa protein, and the gene consists of 11 exons and 10 introns and spans ~13.45 kb of genomic DNA (24). Approximately 180 SNPs in this gene are reported in the dbSNP build 37.1 database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?showRare=on&chooseRs=all&go=Go&locusId=7127). Of the 30 SNPs in the 3' UTR of *TNFAIP2*, 15 are located in the predicted miRNAs-binding sites (29). However, only four known SNPs [rs8126 C allele (miR-184), rs710100 A allele (miR-155), rs1052912 A allele (miR-105) and rs1052823 T allele (miR-550)] are common (i.e. minor allele frequency >0.05) (Figure 1A). In the present study, we tested the hypothesis that genetic variants of these selected SNPs of *TNFAIP2* are associated with risk of SCCHN. To address the functional

Fig. 1. *TNFAIP2* gene structure, reporter gene constructs for the *TNFAIP2* 3' UTR and luciferase expression of the constructed 3' UTR in different cell lines. (A) The four validated SNPs and their exact locations within the *TNFAIP2* 3' UTR. The sequence complementarity of *miR-184* and *TNFAIP2* (rs8126) is shown here. The SNP rs8126 T > C is located within the seed region of the binding site with the C allele perfectly matching the corresponding G allele in *miR-184*; (B) schematic drawing of the reporter gene constructs containing a 430 bp *TNFAIP2* 3' UTR region; the only difference between the two constructs was a T or C at the stop codon 1872 polymorphic site and (C) luciferase activity of the two *TNFAIP2* 3' UTR constructs in H1993 (lung cancer cell line), UM-SCC-1 and MDA686LN (head and neck cell lines). All constructs were co-transfected with pRL-TK-Renilla plasmid as internal control. For each cell line, fold induction of the relative luciferase activity was measured by setting reporter gene activity of construct with pMIR-REPORTER plasmid as 1, using the data (mean $\pm$ SD) from three independent transfection experiments.

relevance of the selected SNPs, we also evaluated the luciferase activity of *TNFAIP2* (rs8126 T and C alleles) by the cloning and reporter gene assay and expression levels of *TNFAIP2* mRNA and related miRNA by the real-time quantitative reverse transcription (RT)-polymerase chain reaction (PCR) assay in peripheral blood mononuclear cells (PBMCs) from SCCHN patients.

Materials and methods

Study subjects

Comprehensive details of cases and controls for SCCHN have been previously reported (20,30). Briefly, the study population included 1077 non-Hispanic White subjects with newly diagnosed untreated primary tumors of the oral cavity ($n = 315$; 29.2%), oropharynx ($n = 549$; 51.0%) and larynx and hypopharynx ($n = 213$; 19.8%) seen at our center during the period from October 1999 to October 2007. By using frequency matching on age (± 5 years), sex and ethnicity, we identified 1073 cancer-free control subjects from among hospital visitors at The University of Texas M. D. Anderson Cancer Center during the same time period. Patients with second SCCHN primary tumors, primary

tumors 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 1077 cases, 64 subjects who had leftover frozen PBMCs and had different genotypes for SNPs included in our study were selected for evaluating mRNA and miRNA expression levels. In addition, we also compared the genotype frequencies of the rs8126 in four populations. Genomic DNA was obtained from 112 African-Americans, 122 Hispanic-Americans and 124 healthy native Chinese donors, who did not have cancer, for evaluation of ethnic differences. These African-American and Hispanic-American subjects were blood donors and participants in cancer screening programs at M. D. Anderson; the Chinese subjects were control subjects in a population-based case-control study conducted in Huai-an and Jin-tan counties, in central Jiangsu province, the People's Republic of China, as described in detail elsewhere (31). Our Institutional Review Board approved the research protocol.

Genotyping of *TNFAIP2*

We extracted genomic DNA from the buffy coat fraction of the blood samples by using a blood DNA mini kit (QIAGEN, Valencia, CA) 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 *TNFAIP2* (rs710100 G > A and rs1052912 G > A) 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. Because the TaqMan assay could not be designed for the rs8126 T > C and rs1052823 G > T polymorphisms, the PCR-restriction fragment length polymorphism method was used to amplify the polymorphisms of rs8126 T > C and rs1052823 G > T. The sequences of the primers used for the genotyping assays were as follows: 5'-GGGGCCGGCTCTCTTGGGCC-3' and 5'-CACACGTACAAA-GACCTTGGGCATCC-3' for rs8126 and 5'-CCTTCGGACTCAGGCAT-GACTC-3' and 5'-GTAAGGCAGTACCTGGGAAAAGGGTA-3' for rs1052823. The PCR profile included an initial melting step of 95°C for 5 min, 35 cycles of 95°C 30 s, 60°C for 45 s and 72°C for 1 min and a final extension step of 72°C for 10 min. These primers generated PCR products of 105 and 108 bp that were digested by the *ApaI* and *RsaI* (New England Biolabs, Beverly, MA), respectively, to identify genotypes for the rs8126 T > C and rs1052823 G > T. The samples from cases and controls were randomly run for the PCR testing on the same plates. Approximately 1.56% (17/1090) of cases and 1.19% (13/1090) of controls did not amplify. The genotypes of rs8126 T > C and rs1052823 G > T were confirmed by direct sequencing (Supplementary Figure 1 is available at *Carcinogenesis* Online). For all genotypes, the assay success rate was >99% and the repeated sample's results were 100% concordant.

Real-time RT-PCR for expression level of *TNFAIP2* mRNA

The expression levels of *TNFAIP2* mRNA were examined by quantitative RT-PCR using total RNA that was isolated from PBMCs of 64 SCCHN patients with the TRIzol reagent (Invitrogen™, Carlsbad, CA). *TNFAIP2* mRNA expression levels were detected by using TaqMan gene expression assays with the master mix reagent (Applied Biosystems) according to the manufacturer's instructions. Each amplification reaction was performed in a final volume of 5 μ l containing 5 ng of the complementary DNA, 0.25 μ l primers and 2.5 μ l Master mix. Real-time RT-PCR was performed using the ABI Prism 7900HT Sequence Detection (Applied Biosystems). 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. The *18S* expression was assessed as an internal control to account for differences of complementary DNA. Each sample was analyzed in duplicate, and if the coefficient of variation of all reactions was <5%, the mean values of the duplicates were used. When coefficient of variation is $\geq 5\%$, the assay will be repeated and the samples with new coefficient of variation $\geq 5\%$ will be removed. The expression level of *TNFAIP2* relative to that of *18S* was calculated using the equation $\text{ratio} = \frac{C_{T\text{TNFAIP2}}}{C_{T18S}} \times 100\%$ (32) to normalize the results to the endogenous *18S* mRNA expression level. In general, the higher normalized Ct values represent the lower gene expression levels.

miRNA quantification by the real-time quantitative RT-PCR

Analysis of *miR-184* expression relative to a *cel-mir-39* non-coding RNA control was performed using TaqMan MicroRNA real-time PCR assay primer sets and reagents (Applied Biosystems), based on absolute quantification,

following the manufacturer's instructions. Each amplification reaction was performed in a final volume of 5 μ l containing 5 ng of the complementary DNA, 0.25 μ l primers and 2.5 μ l Master mix. Real-time PCR was performed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). 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 60 s. Each sample was tested in duplicate. The amount expression levels of *miR-184* relative to *cel-mir-39* small nuclear RNA was calculated using the equation $\text{ratio} = C_{t_{\text{miR-184}}} / C_{t_{\text{cel-mir-39}}} \times 100\%$ (32).

Cloning and site-directed mutagenesis

The 430 bp human *TNFAIP2* 3' UTR containing the T allele at rs8126 was amplified with the forward primer 5'-GTAGACGCGTATCTTTGGGAGG-CGGTATT-3' and the reverse primer 5'-TCCAAAGCTTAAAAACCTGGA-TCCTGTTC-3' from a homozygous human genomic DNA sample. The PCR products were separated in agarose gel and extracted, purified and cloned into pMIR-REPORT plasmids (Applied Biosystems) with *Mlu*I and *Hind*III digestion. The pMIR-REPORT miRNA expression reporter vector system consists of an experimental firefly luciferase reporter vector and an associated β -gal reporter control plasmid. By inserting predicted miRNA target sequences in the multiple cloning sites, the plasmids can be used to conduct accurate, quantitative evaluation of miRNA function. In this study, the C allele of rs8126 was generated with the QuickChange Site-Directed Mutagenesis kit (Cat# 200518; Stratagene, La Jolla, CA) with forward mutagenic primer 5'-CTCTCTTGGGTCCGTCCTCCCTTTTCCAGGTACTGC-3' and reverse mutagenic primer 5'-GCAGTACCTGGGAAAGGGGACGGACCAAGA-GAG-3' according to the manufacturer's protocol. This method was used to avoid non-specific cloning during the construction of mutant libraries (33). All constructs used in this study were verified by directing sequencing (Figure 1B).

Transient transfection and luciferase assay

The head and neck cancer cell lines UM-SCC-1 and MDA686LN and lung cancer cell line H1993 (from the collection in the Department of Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA) were cultured in Dubecco's modified Eagle's medium or RPMI 1640 medium, supplemented with 10% fetal bovine serum (Sigma, St Louis, MO), 1% L-glutamine (InvitrogenTM) and 1% penicillin-streptomycin (InvitrogenTM) at a 37°C incubator supplemented with 5% CO₂. A total of 0.5×10^5 each cell line was plated onto each well of 24-well plates (BD Biosciences, Bedford, MA), and 24 h after the plating, cells were co-transfected with the FuGENE HD reagent (Roche Applied Science, Indianapolis, IN). Each co-transfection reaction contained 200 ng of pMIR-*TNFAIP2* (rs8126) T or C vector plus 50 ng pRL-TK (Promega, Madison, WI) plasmids that served as a transfection internal control. Forty-eight hours after transfection, cells were washed and lysed with 100 μ l Passive Lysis Buffer (Promega). The luciferase activities of both firefly and renilla luciferase were quantified by a Dual-Luciferase Reporter Assay System (Promega), and the relative luciferase activity was obtained, according to the manufacturer's instructions (BD Monolight(tm) 3010 Luminometer; Becton Dickinson Company, Mississauga, ON). Independent triplicate experiments were done for all samples.

Statistical analysis

The differences in selected demographic variables, smoking and alcohol consumption between SCCHN cases and controls were evaluated by using the chi-square test. The associations of genotypes of *TNFAIP2* with risk of SCCHN were estimated by computing the odds ratios (ORs) and their 95% confidence intervals (CIs) from both univariate and multivariate logistic regression models in case-control analysis. These analyses were performed with or without adjustment for age (in years), sex, pack-years and alcohol consumption. The associations of *TNFAIP2* genotypes with SCCHN risk were also stratified by age, sex, smoking and drinking status, tumor site and tumor stage. Those subjects who had smoked >100 cigarettes in their lifetime were defined as ever smokers as traditionally used in epidemiological studies; those who had quit smoking for >1 year previously were considered as former-smokers and the rest were considered current-smokers. Subjects who drank alcoholic beverages at least once a week for >1 year in previous years were defined as ever drinkers; of these, those who had quit drinking for >1 year previously were defined as former drinkers and the others as current drinkers. Pack-years smoked [(cigarettes per day/20) $\times$ years smoked] were calculated to indicate the cumulative smoking dose and average drinks per week were used to evaluate the alcohol consumption. The mean and standard error of fold induction in expression were calculated and tested with the Student *t*-test. We applied the PROC HAPLOTYPE procedure in SAS/Genetics software to infer haplotype frequencies among the four variants of *TNFAIP2* based on their observed genotypes. We used the false-positive report probability (FPRP) to test for false-positive associations (34). 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, Cary, NC), unless stated otherwise.

Results

The distributions of selected characteristics of the cases and controls are presented in Table I. There were no significant differences in the distributions of age and sex between the cases and the controls with similar mean ages of 57.1 (± 11.2) years for cases and 56.7 (± 11.0) years for controls. The cases had more current-smokers (37.6 versus 14.6%) or current drinkers (50.9 versus 40.7%) than the controls. Furthermore, the differences in pack-years and alcohol consumption were significant between cases and controls (both $P < 0.001$). However, all these variables were further adjusted for any residual confounding effect in later multivariate logistic regression analysis.

The genotype and allele frequencies of the rs8126 T > C, rs710100 G > A, rs1052912 G > A and rs1052823 G > T SNPs and their associations with risk of SCCHN are summarized in Table II. The genotype distribution of the four SNPs in the controls was in agreement with the Hardy-Weinberg equilibrium ($P = 0.717$ for rs8126, 0.821 for rs710100 and 0.365 for rs1052823), except for rs1052912 ($P = 0.003$). Compared with the rs8126 TT genotype, the variant CC and CC/CT genotypes were associated with a statistically significantly increased risk of SCCHN (adjusted OR, 1.48; 95% CI, 1.06–2.05; $P = 0.020$ for the CC genotype and adjusted OR, 1.23; 95% CI, 1.03–1.47; $P = 0.024$ for CC/CT variant genotypes; $P_{\text{trend}} = 0.009$). These risks remained in patients with oropharyngeal cancers but not with non-oropharyngeal cancers. No associations were observed between the genotypes of other SNPs (rs710100 G > A, rs1052912 G > A and rs1052823 G > T) and SCCHN risk either overall or by sub sites (Table II). In addition, we conducted haplotype analysis for the four SNPs in *TNFAIP2* and no significant associations were found (data not shown). In the stratified analysis with the rs8126 T > C SNP (Table III), the increased risk associated with the variant CC/CT genotypes was more evident among older subjects (adjusted OR = 1.40; 95% CI, 1.08–1.82) and light drinkers (alcohol consumption ≤ 8.0 per week) (adjusted OR = 1.76; 95% CI, 1.26–2.45), compared with the TT genotype.

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 IV, when the assumption of prior probability was 0.25, the association with *TNFAIP2* (rs8126) CC genotype was still noteworthy for all subjects (FPRP = 0.024) as well as for age >57 years (FPRP = 0.033) and alcohol <8.0 (FPRP = 0.014).

We have evaluated the genotype frequencies of the rs8126 in cancer-free healthy subjects of four ethnic groups, including 1073 non-Hispanic Whites, 112 African-Americans, 122 Hispanic-Americans and 124 healthy native Chinese donors, who had frequency of genotype TT, CT and CC for *TNFAIP2* (rs8126) of 51.1, 41.1 and 7.8% for White, respectively; 38.5, 52.5 and 9.0% for Hispanic-Americans, respectively; 38.4, 50.0 and 11.6% for African-Americans, respectively and 44.4, 41.9 and 13.7% for native Chinese, respectively. The differences of genotypes and alleles distributions for four populations are significant ($P = 0.009$ and $P < 0.001$, respectively) (Supplementary Table I is available at *Carcinogenesis* Online).

As shown in Figure 1C, significantly lower levels of luciferase expression were observed when co-transfected *TNFAIP2* 3' UTR luciferase reporter plasmids carried the rs8126 C allele, compared with the that carried the T allele (H1993 cell line: 0.644 ± 0.078 for C allele versus 0.894 ± 0.079 for the T allele, $P = 0.018$; UM-SCC-1 cell line: 0.365 ± 0.028 for C allele versus 0.777 ± 0.094 for the T allele, $P = 0.011$; MDA686LN, 0.438 ± 0.036 for C allele versus 0.681 ± 0.005 for the T allele, $P < 0.001$).

To further characterize biological significance of the *TNFAIP2* rs8126, we conducted correlation analysis between rs8126 genotypes and expression levels of *TNFAIP2* mRNA in PBMCs from 64 SCCHN

Table I. Frequency distributions of selected variables in SCCHN cases and cancer-free controls recruited from M. D. Anderson Cancer Center in Houston, TX between October 1999 and October 2007

Variables	Cases (<i>n</i> = 1077)	Controls (<i>n</i> = 1073)
	<i>n</i> (%)	<i>n</i> (%)
Age (years) ^a		
≤57 (median)	576 (53.5)	560 (52.2)
>57 (median)	501 (46.5)	513 (47.8)
Sex		
Female	267 (24.8)	251 (23.4)
Male	810 (75.2)	822 (76.6)
Smoking status		
Never	302 (28.0)	523 (48.7)
Former	370 (34.4)	394 (36.7)
Current	405 (37.6)	156 (14.6)
Pack-years ^a		
None	302 (28.0)	523 (48.7)
≤21.0 (median)	235 (21.8)	282 (26.3)
>21.0 (median)	540 (50.2)	268 (25.0)
Alcohol use		
Never	293 (27.2)	463 (43.2)
Former	236 (21.9)	173 (16.1)
Current	548 (50.9)	437 (40.7)
Alcohol consumption ^a (average drinks per week)		
None	293 (27.2)	463 (43.2)
≤8.0 (median)	276 (25.6)	325 (30.3)
>8.0 (median)	508 (47.2)	285 (26.6)
Tumor site		
Oropharynx	549 (51.0)	
Non-oropharynx ^b	528 (49.0)	

^aThe median values in controls were used as the cutoff.^bIncluded oral cavity (*n* = 315), larynx (*n* = 170) and hypopharyngeal (*n* = 43).

patients. We found that, in the 64 SCCHN cases, 20 had the rs8126 CC genotype, 21 had the CT genotype and 23 had the TT genotype. As shown in Figure 2A, the normalized Ct values (%) of *TNFAIP2* to *18S* was higher for the CC genotype ($216.13 \pm 15.94\%$) than that for the TT genotype ($206.94 \pm 11.45\%$), and this difference was statistically significant ($P = 0.034$). In general, higher normalized Ct values represent less expression levels. In other words, the cases with the rs8126 CC genotype, which has the perfect complementary seed sequence for *miR-184*, had lower levels of *TNFAIP2* expression than did cases with the TT genotype. Although the trend test for the effect of the C allele on the expression was toward significance ($P_{\text{trend}} = 0.082$), the normalized Ct values (%) for the CT genotype ($211.40 \pm 22.61\%$) was not significantly different from that for the TT genotypes ($P = 0.423$) nor was the normalized Ct values (%) for the CT/CC genotypes (213.71 ± 19.54) significantly different from that for the TT genotype ($P = 0.086$). Because rs710100 G > A, rs1052912 G > A and rs1052823 G > T genotypes were not associated with SCCHN risk, their related miRNAs were not pursued in further laboratory studies.

To exclude the possibility that patients with different *TNFAIP2* rs8126 T > C genotypes may have different *miR-184* levels that would contribute to the differential *TNFAIP2* expression levels; we used RT-PCR to measure the *miR-184* expression levels in PMBCs from the same 64 SCCHN patients. We found that the *cel-mir-39* and *miR-184* were detectable in all samples that were both highly concordant in duplicate. As shown in Figure 2B, there were no significant difference in the normalized Ct values (%) of *miR-184* among CC, CT and TT genotypes (TT versus CC: $132.36 \pm 20.53\%$ versus $137.05 \pm 17.74\%$; TT versus CT: $132.36 \pm 20.53\%$ versus $133.24 \pm 7.86\%$; and TT versus CC/CT: $132.36 \pm 20.53\%$ versus $135.10 \pm 13.57\%$; $P = 0.430, 0.849$ and 0.569 , respectively).

Discussion

In this hospital-based case-control study of 1077 patients with SCCHN and 1073 cancer-free controls in a non-Hispanic White

Table II. Genotype frequencies of the *TNFAIP2* polymorphisms among SCCHN cases and control subjects and their associations with SCCHN risk in a case-control study conducted at M. D. Anderson Cancer Center in Houston, TX between October 1999 and October 2007

Genotypes	Controls (%)	Overall		Oropharynx		Non-oropharynx ^a	
		Cases (%)	OR (95% CI) ^b	Cases (%)	OR (95% CI) ^b	Cases (%)	OR (95% CI) ^b
All subjects	1073 (100.0)	1077 (100.0)		549 (100.0)		528 (100.0)	
<i>rs8126</i> T > C							
TT	548 (51.1)	488 (45.3)	1.00	249 (45.4)	1.00	239 (45.3)	1.00
CT	441 (41.1)	483 (44.9)	1.18 (0.98–1.43)	242 (44.1)	1.18 (0.94–1.47)	241 (45.6)	1.22 (0.96–1.55)
CC	84 (7.8)	106 (9.8)	1.48 (1.06–2.05)	58 (10.5)	1.53 (1.04–2.23)	48 (9.1)	1.32 (0.86–2.03)
P_{trend}			0.009		0.022		0.075
CC/CT	525 (48.9)	589 (54.7)	1.23 (1.03–1.47)	300 (54.6)	1.24 (1.01–1.54)	289 (54.7)	1.24 (0.98–1.56)
<i>rs710100</i> G > A							
GG	515 (48.0)	492 (45.7)	1.00	254 (46.3)	1.00	238 (45.1)	1.00
AG	459 (42.8)	474 (44.0)	1.05 (0.87–1.27)	234 (42.6)	1.02 (0.81–1.28)	240 (45.5)	1.12 (0.88–1.42)
AA	99 (9.2)	111 (10.3)	1.22 (0.89–1.67)	61 (11.1)	1.29 (0.90–1.86)	50 (9.5)	1.12 (0.74–1.69)
P_{trend}			0.242		0.279		0.396
AA/AG	558 (52.0)	585 (54.3)	1.08 (0.90–1.29)	295 (53.7)	1.07 (0.86–1.32)	290 (54.9)	1.12 (0.89–1.41)
<i>rs1052912</i> G > A							
GG	858 (80.0)	831 (77.2)	1.00	423 (77.1)	1.00	408 (77.3)	1.00
AG	213 (19.8)	238 (22.1)	1.13 (0.91–1.41)	123 (22.4)	1.14 (0.88–1.48)	115 (21.7)	1.13 (0.85–1.49)
AA	2 (0.2)	8 (0.7)	4.35 (0.87–21.81)	3 (0.5)	3.10 (0.50–19.43)	5 (1.0)	4.82 (0.69–33.84)
P_{trend}			0.108		0.203		0.212
AA/AG	215 (20.0)	246 (22.8)	1.16 (0.94–1.44)	126 (22.9)	1.16 (0.90–1.50)	120 (22.7)	1.16 (0.88–1.53)
<i>rs1052823</i> G > T							
GG	852 (79.4)	830 (77.1)	1.00	422 (76.9)	1.00	408 (77.3)	1.00
GT	205 (19.1)	228 (21.2)	1.12 (0.89–1.39)	115 (20.9)	1.09 (0.84–1.43)	113 (21.4)	1.13 (0.85–1.50)
TT	16 (1.5)	19 (1.7)	1.35 (0.66–2.73)	12 (2.2)	1.65 (0.75–3.66)	7 (1.3)	0.98 (0.37–2.64)
P_{trend}			0.227		0.243		0.479
TT/GT	221 (20.6)	247 (22.9)	1.13 (0.91–1.40)	127 (23.1)	1.13 (0.87–1.46)	120 (22.7)	1.12 (0.85–1.48)

^aIncluded oral cavity (*n* = 315), larynx (*n* = 170) and hypopharyngeal (*n* = 43).^bAdjusted for age, sex, pack-years and alcohol consumption in a logistic regression model.

Table III. SCCHN risk associated with *TNFAIP2* rs8126 genotypes by selected subject characteristics in a case-control study conducted at M. D. Anderson Cancer Center in Houston, TX between October 1999 and October 2007

Variables	Cases (<i>n</i> = 1077)		Controls (<i>n</i> = 1073)		Adjusted OR (95% CI) ^a		<i>P</i> ^a
	TT <i>n</i> (%)	CC/CT <i>n</i> (%)	TT <i>n</i> (%)	CC/CT <i>n</i> (%)	TT	CC/CT	
Age (years)							
≤57	268 (46.5)	308 (53.5)	280 (50.0)	280 (50.0)	1.00	1.10 (0.86–1.40)	0.445
>57	220 (43.9)	281 (56.1)	268 (52.2)	245 (47.8)	1.00	1.40 (1.08–1.82)	0.012
Sex							
Female	134 (50.2)	133 (49.8)	147 (58.6)	104 (41.4)	1.00	1.26 (0.86–1.83)	0.232
Male	354 (43.7)	456 (56.3)	401 (48.8)	421 (51.2)	1.00	1.21 (0.99–1.48)	0.067
Pack-years							
None	144 (47.7)	158 (52.3)	284 (54.3)	239 (45.7)	1.00	1.30 (0.98–1.74)	0.071
≤21.0	113 (48.1)	122 (51.9)	133 (47.2)	149 (52.8)	1.00	1.03 (0.72–1.47)	0.880
>21.0	231 (42.8)	309 (57.2)	131 (48.9)	137 (51.1)	1.00	1.30 (0.96–1.76)	0.091
Alcohol consumption (average drinks per week)							
None	144 (49.2)	149 (50.8)	221 (47.7)	242 (52.3)	1.00	0.94 (0.70–1.27)	0.700
≤8.0	118 (42.8)	158 (57.2)	185 (56.9)	140 (43.1)	1.00	1.76 (1.26–2.45)	0.001
>8.0	226 (44.5)	282 (55.5)	142 (49.8)	143 (50.2)	1.00	1.19 (0.88–1.61)	0.262

^aAdjusted for age, sex, pack-years and alcohol consumption in a logistic regression model, where it was appropriate.

Table IV. FPRP values for associations between risk of SCCHN and frequencies of rs8126 genotypes

Genotype	Positive OR 95% CI ^a	<i>P</i> ^b	Statistical power ^c	Prior probability				
				0.25	0.1	0.01	0.001	0.0001
rs8126 T > C	TT versus CC/CT							
All subjects	1.26 (1.06–1.49)	0.008	0.979	0.024	0.069	0.447	0.891	0.988
Oropharynx	1.26 (1.02–1.55)	0.029	0.672	0.084	0.215	0.751	0.968	0.997
Age > 57 years	1.40 (1.09–1.79)	0.008	0.713	0.033	0.092	0.526	0.918	0.991
Alcohol ≤ 8.0	1.77 (1.28–2.45)	0.001	0.204	0.014	0.042	0.327	0.830	0.980

^aThe crude OR.

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

^cCalculated using study subjects to detect an OR of 1.50 with the common genotype used as the reference.

population, we evaluated the associations between four common polymorphisms at the miRNA-binding sites of the *TNFAIP2* gene and risk of SCCHN. We found that the rs8126 variant CC and CC/CT genotypes were associated with a significantly increased risk of SCCHN compared with the TT genotype. This association was not dramatically different across the strata by different subgroups such as age, sex, smoking and drinking, although the risk tended to be more pronounced in older subjects, light drinkers and those who had oropharyngeal cancer. We also provided biological evidence that the expression levels of *TNFAIP2* are regulated by the 3' UTR genotypes (rs8126 variant CC and CC/CT) at the polymorphic miRNA-binding site in both lymphocytes and cancer cell lines. Additional functional analyses showed that the rs8126 variant C allele led to significantly lower luciferase activity, compared with the T allele. These data suggest that the miRNA-binding site polymorphism (rs8126) may mediate the expression levels of *TNFAIP2* and thus play a role in the etiology of SCCHN.

Our study was a hospital-based case-control study and the control were not selected from the same population from which the cases may occur. Although our study had over 1000 SCCHN cases and 1000 controls, our sample size was still not large enough to identify significant associations in some subgroups. These issues need to be further addressed and validated in studies with different cancer sites and large sample sizes, including different ethnic groups. Also, due to the retrospective nature of the original study design, we did not have reliable information on human papillomavirus infection that most likely cause oropharyngeal cancer, and we do not know exactly how the rs8126 variant works to influence the risk of SCCHN.

miRNAs regulate gene expression by the sequence-specific binding to target mRNA (35), but the binding affinity may be affected by SNPs residing in miRNA target sites, which may in turn affect the miRNAs' ability to inhibit the mRNA translation into proteins or lead to degradation of the mRNA (36). Indeed, recent studies have demonstrated that SNPs at miRNA-binding sites are likely to affect expression of the miRNA target genes and thus may contribute to susceptibility to cancer (16,17,32,35). Many studies have also shown that SNPs at miRNA-binding sites play a role as novel biomarkers for cancer risk, earlier diagnosis of cancer and clinical outcome. For example, in the study of 697 colorectal cancer patients and 624 healthy Caucasian population controls, Landi *et al.* (17) found that the variant alleles at the miRNA-binding site in the CD86 gene were associated with a significantly increased risk of colorectal cancer. In another study of 1110 breast cancer cases and 1097 cancer-free controls in a Chinese population, Song *et al.* (32) have found that the miR-502-binding site SNP in the 3' UTR of *SET8* modulates the SET8 expression and contributes to the early development of breast cancer.

To date, few studies have investigated the effect of SNPs at the miRNA-binding sites on head and neck cancers. In one case-control study of 560 oral cancer cases and 500 cancer-free controls in a Caucasian population, the authors found no association of the let-7-binding site SNP in the 3' UTR region of *KRAS-LCS6* with an overall risk of developing oral cancer, but this study did find the *KRAS-LCS6* variant genotypes to be associated with poor prognosis in patients with oral cancers (37). Another relatively small study (150 cases and 300 controls) reported that the variant genotype of a miRNA-binding site SNP (rs3747238) in *SMC1B* was associated with

Fig. 2. Relative expression levels of *TNFAIP2* mRNA and *miR-184* in PBMCs of 64 SCCHN cases with known *TNFAIP2* (rs8126) genotypes. Quantitative real-time RT-PCR was used to measure the relative expression of *TNFAIP2* mRNA or *miR-184*. Housekeeping gene *18S* or *cel-mir-39* was used as the reference. The threshold cycle or Ct value is the PCR cycle at which a significant increase in fluorescent signal is first detected. Ct values form the basis for quantitative comparison of genes in the real-time PCR. The smaller the Ct value, the higher the level of the measured target RNA. We normalized the relative levels of *TNFAIP2* to that of *18S* using the equation $\text{ratio} = C_{tTNFAIP2}/C_{t18S} \times 100\%$ and the relative levels of *miR-184* to that of *cel-mir-39* using the equation $\text{ratio} = C_{tmiR-184}/C_{tcel-mir-39} \times 100\%$. A higher normalized Ct value represents less expression. (A) The TT genotype had lower normalized Ct values and, thus, higher *TNFAIP2* mRNA expression levels, whereas the CC genotype had higher relative Ct values, representing lower expression levels in PBMCs of SCCHN cases ($P = 0.034$). However, Ct values of the CT genotype was not significantly different from that of the TT genotype ($P = 0.423$). (B) The expression level of *miR-184* was measured by real-time PCR in PBMCs of 64 SCCHN cases. The expression levels are similar among the three groups with the *TNFAIP2* CC, CT and TT genotypes ($P = 0.430$ for CC versus TT and $P = 0.849$ for CT versus TT).

significantly increased risk of second primary tumors/recurrence in head and neck cancer patients (38). However, no reported studies have investigated the association between *TNFAIP2* rs8126 T > C genotypes and cancer risk.

The *TNFAIP2* gene may play a role in apoptotic response by induction of *TNFAIP2* mRNA expression, notably in hematopoietic and lymphoid tissues (39). Even with its still largely unknown function, the *TNFAIP2* gene has been identified as an apoptosis-related gene targeted by retinoic acid, and this gene has been found to play a potential role in acute pro-myelocytic leukemia and other cancers (25–27). More recently, Chen *et al.* (28) found that the *TNFAIP2* mRNA and protein were highly expressed in NPC tumor cells; the increased *TNFAIP2* expression was associated with shorter distant metastasis-free survival in NPC patients.

Our analysis of the correlation between rs8126 genotypes and the expression levels of *TNFAIP2* mRNA suggests that the relative expression levels of *TNFAIP2* to *18S* were not significantly different by the rs8126 genotypes in 37 SCCHN cell lines (data not shown); however, for blood lymphocytes from the 64 SCCHN cases, the relative (mRNA) expression levels of *TNFAIP2* to *18S* were significantly higher for the CC genotype, but not the CT genotype, than for the TT genotype and the effect of the C allele was toward a trend by the number of the alleles. It is likely that in tumor tissues, such as SCCHN cancer cell lines, additional somatic changes may have overwhelmed any effects of SNPs at the miRNA-binding sites in the *TNFAIP2* gene, whereas such a possible event is absent in normal tissue, such as the unaffected blood lymphocytes. We were investigating the role of SNPs in the miRNA-binding sites of *TNFAIP2* on the expression of the gene, and we did not observe overexpression of the miRNA. However, it is likely that if the SNP/variant affects the binding affinity, then the overexpression of *miR-184* in SCCHN could occur as a compensation for a weakened binding affinity, which not necessarily has much impact on the *TNFAIP2* regulation. Therefore, the SNP at the binding site, not the level of miRNA, appeared to regulate the gene expression, as our data suggested.

miR-184 is a single-copy gene and evolutionarily conserved at the nucleotide level from flies to humans. Although the function of *miR-184* remains unknown, it is predicted to target several hundred genes. For example, Weitzel *et al.* (40) found that *miR-184* regulated *NFAT1* expression in umbilical cord blood CD4⁺ T cells and that the capability of *miR-184* to regulate NFAT1 protein expression did not cause transcript degradation through its predicted complementary binding site within the *NFATc2* mRNA 3' UTR. Some studies reported that *miR-184* caused a decrease in cell numbers and an increase in apoptosis in neuroblastoma cell lines (41). Foley *et al.* (9) observed that *miR-184* inhibits neuroblastoma cell survival through targeting the serine/threonine kinase AKT2. So far, few studies have investigated the level of expression of *miR-184* as a risk factor for cancer. Yu *et al.* (42) observed that *miR-184* appears to have a tumor suppressive effect in SCC cell lines. However, this result seems contradictory to another paper published by Wong *et al.* (8), in which *miR-184* was found to play an important role in the anti-apoptotic and proliferative processes of tongue SCC, and *miR-184* levels in tongue SCC patient plasma were found to be associated with the presence of a primary tumor.

In the current study, in contrast to the correlation between expression of *TNFAIP2* and the SNPs at its miRNA-binding sites, there was no difference between the *miR-184* expression levels and *TNFAIP2* (rs8126) genotypes in the blood lymphocytes, suggesting that the expression of miRNA itself may not be affected by SNPs at the binding sites in the target genes. Our results indicate that the *TNFAIP2* (rs8126) C allele, located in the *miR-184* binding site, is likely to disrupt miRNA target interaction, resulting in the alteration of *TNFAIP2* mRNA expression, a possible underlying mechanism for the observed association with increased risk of SCCHN. However, the exact effect of rs8126 on the binding between *miR-184* and *TNFAIP2* still need further investigation by functional studies.

In summary, we found some evidence of an association between the *TNFAIP2* (rs8126) polymorphism at the miRNA-binding site and the risk of SCCHN in a non-Hispanic White population in this case-control study. We also observed that the *TNFAIP2* (rs8126) CC

genotype was associated with significantly reduced expression of *TNFAIP2* mRNA in normal lymphocytes from patients but had no effect on the expression of miRNAs. Further functional characterization of miRNA SNPs and their influences on target mRNAs may provide underlying mechanisms for the observed associations and disease etiology. However, the FPRP values for most of all the significant findings in our study were <0.05 only when the prior probability was set at 0.25, suggesting that these findings may not be noteworthy. Therefore, additional larger replication studies are needed to confirm these results.

Supplementary material

Supplementary Figure 1 and Table 1 can be found at <http://carcin.oxfordjournals.org/>

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