The Modifying Effect of a Functional Variant at the miRNA Binding Site in E2F1 Gene on Recurrence of Oropharyngeal Cancer Patients with Definitive Radiotherapy

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Abstract

Human papillomavirus (HPV) activates E2F1-driven transcription via the E7-RB-E2F1 pathway. A polymorphism in the 3′ UTR of E2F1 gene may disrupt a binding site for miRNA and may affect its transcription level, thus modifying the susceptibility to radiotherapy and outcomes through this pathway. We evaluated the association of a polymorphism at the 3′ UTR miRNA binding site of E2F1 gene (rs3213180) with risk of recurrence of SCCOP in a cohort of 1008 patients. Log-rank test and univariate and multivariable Cox models were used to evaluate the associations. Compared with patients with E2F1-rs3213180 GG homozygous genotype, the patients with E2F1-rs3213180 GC + CC variant genotypes had significantly better disease-free survival (log-rank P < .001) and decreased risk of SCCOP recurrence (HR, 0.4, 95% CI, 0.3–0.5) after multivariable adjustment. Furthermore, among patients with HPV16-positive tumors, the patients with E2F1-rs3213180 GC + CC variant genotypes had significantly better disease-free survival rates (log-rank P < .001) and lower recurrence risk than those with E2F1-rs3213180 GG homozygous genotype (HR, 0.2, 95% CI, 0.1–0.4). Our findings suggest that E2F1-rs3213180 polymorphism may modulate the risk of recurrence in SCCOP patients, particularly for patients with HPV16-positive tumors of SCCOP. However, future larger population and functional studies are warranted to validate these results.

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Introduction

Oral squamous cell carcinoma (OSCC), a subsite of squamous cell carcinoma of the head and neck (SCCHN), includes squamous cell carcinoma of the oropharynx (SCCOP) and oral cavity squamous cell carcinoma (OCSCC). Over the past three decades, the incidence of SCCHN continues decreasing, partially due to the success of public health efforts in tobacco control [1,2], while the incidence of SCCOP

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is increasing [3] due to the increasing infection of human papillomavirus (HPV). The patients with SCCOP are characterized with local tumor aggressiveness, the high frequency of second primary malignancies, the high recurrence rate moderately, and a high frequency of medical comorbidities [4]. Surgery, radiotherapy, and chemotherapy have been used individually or in combination to treat SCCOP successfully, however disease recurrence remains a major problem clinically for mortality. The recurrence rates of SCCOP are not consistent among patients who have similar clinical characteristics and similar therapeutic approaches. Clinicians would ensure appropriately individualized treatment if they could accurately predict the recurrence risk of individual patients with SCCOP for improved survival and better life quality.

As HPV has been considered as an etiologic risk factor for SCCOP, approximately 50–80% of SCCOP patients are likely to be HPV-positive [HPV (+)] [5,6]. Among the types of HPV, HPV16 is the most common high-risk type of HPV in SCCOP, accounting for approximately 90–95% of HPV (+) SCCOP [6,7]. Smoking and HPV are well known factors for prognosis of SCCOP, while the prognosis is different for SCCOP patients with similar clinical characteristics and similar treatment, suggesting other factors, such as polymorphism, may play a role in prognosis.

The E2F1 gene is one of key members of the E2F family, locating on chromosome 20q. E2F1 regulates expression of genes in many molecular pathways including cell cycle progression, apoptosis and DNA synthesis [8]. Previous studies showed that E2F1 had been demonstrated as a key downstream target in the retinoblastoma (RB) pathway [9,10]. Binding of E7 to phosphorylated RB leads to activation of E2F1, which induces cell-cycle progression and p16 expression [11]. Therefore, E2F1 and HPV may interact to activate gene transcription via the E7-RB-E2F pathway. Furthermore, down expression of E2F1 enhanced tumor growth in HPV(+) oral cancer, while overexpression of E2F1 decreased the clonogenicity of HPV(+) cancer [12].

MiRNA plays important roles in many ranges of biological processes. MiRNAs can bind to targeted mRNA in the 3’ UTR to alter mRNA levels and expression of proteins. Thus genetic variants in the 3’ UTR targeted by miRNAs can either create illegal binding sites or remove existing binding sites, affecting the regulation of target genes [13–15]. Such functional variants at the miRNA binding site in E2F1 gene may alter its expression, thus subsequently influencing the response to radiotherapy and susceptibility to recurrence. E2F1-rs3213180, one of such single nucleotide polymorphisms (SNP), was recently reported to be located in the 3’UTR miRNA binding site of the E2F1 gene. E2F1-rs3213180 was significantly associated with E2F1 expression and might alter E2F1 gene function [16], and our previous study has found that E2F1-rs3213180 polymorphism was associated with risk of HPV-associated OSCC, particularly for SCCOP [17]. While no large studies have examined the associations between the E2F1-rs3213180 polymorphism and risk of recurrence of patients with SCCOP, we hypothesized that E2F1-rs3213180 polymorphism at miRNA binding site in 3’ UTR may cause interindividual variations in responses to radiotherapy, leading to different risk of SCCOP recurrence. Therefore, in the present study we evaluated the association in a cohort of 1008 incident patients with SCCOP.

### Study Population

This study included 1008 patients with newly diagnosed, histopathologically confirmed, and untreated SCCOP. These cases had been recruited consecutively at the University of Texas MD Anderson Cancer Center as part of an ongoing molecular epidemiologic study of SCCHN. All patients provided written informed consent to be enrolled in the study, which was approved by The University of Texas MD Anderson Cancer Center’s Institutional Review Board. All informed consent was obtained from all subjects. The committee approved all experiments performed in this study. In addition, all methods were performed in accordance with the relevant guidelines and regulations. Clinical data, including site and histologic subtype of the tumor, stage at presentation of the tumor and any comorbidity, recurrence and therapy, were obtained from review of the medical records. Patients who had smoked over 100 cigarettes during their lifetimes were categorized as ‘ever-smokers’ and others as ‘never-smokers.’ Those who had drunk alcoholic beverages at least once a week for more than 1 year were classified as ‘ever-drinkers’ and otherwise as ‘never-drinkers.’ During enrollment in the study, some participants were excluded for final analysis if they 1) had known distant metastases; 2) had any prior cancer except non-melanoma skin cancer; 3) had a salivary gland tumor, cervical metastases of unknown origin, a primary sinonasal tumor, or a tumor outside the upper aerodigestive tract; 4) had treatment outside of our institution; or 5) experienced only palliative treatment.

Patients were followed up throughout their treatment and posttreatment course, including scheduled regular clinical and radiographic examinations. Patients were considered disease free if there was no documented disease at the last visit with the head and neck surgeon, head and neck radiation oncologist, or head and neck medical oncologist. Recurrent disease was defined as appearance of a new lesion of the same histology verified by biopsy (excisional, incisional, or needle biopsy), reappearance of any lesion that had disappeared, or development of tumor-related symptoms. The sixth edition of the American Joint Committee on Cancer TNM staging system was used to determine disease stage at the time of presentation for all study patients. Patients with comorbidities were categorized into two groups—none or mild and moderate to severe using a modification of the Kaplan-Feinstein comorbidity index (Adult Comorbidity Evaluation 27), as described in our previously report [18].

### E2F1-rs3213180 Genotyping

We extracted genomic DNA from peripheral blood leukocyte cell pellet using the QIamp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer’s instructions. The quantification of DNA was determined by a Nanodrop analyzer (ND-1000 spectrophotometer [Nano Drop Technologies, Inc., Wilmington, DE]). The SNP was genotyped using the TaqMan assay. The PCR amplification was run and the plate was read with the built-in Sequence Detection Software on an ABI-Prism 7900 instrument (Applied Biosystems, Foster City, CA). The analysis was repeated on a randomly selected subset of 10% of the samples, and the repeated test results demonstrated 100% concordance with the original analysis.

### HPV16 Testing

Paraffin-embedded tumor tissue biopsy samples or specimens from SCCOP patients were used to extract DNA for HPV16 detection,
using the PCR-based, type-specific assays for the E6 and E7 regions, as we previously reported [19]. 10% of the samples were retested using restriction digestion of the PCR products with BanII and MspI to verify the presence of E6- and E7-specific fragments. The results of both methods were 100% concordant.

**Statistical Analysis**

Student t-test was used to detect the differences between mean age at diagnosis and follow-up time between patients with and without recurrence. The associations between demographic variables (age at diagnosis, sex, and ethnicity); epidemiologic factors (smoking status and alcohol drinking status); clinical factors (comorbidity group, tumor stage, and treatment); and time to recurrence were assessed using both univariate and multivariable Cox proportional hazards regression models. The estimates of association of each variable with disease-free survival (DFS)/recurrence status were assessed by a log-rank test. Time to recurrence was from the date of SCCOP diagnosis to the date of recurrence. Patients without known recurrence at the date of last follow up, those lost to follow-up, and those who died of any cause were censored. We quantified the associations of E2F1 polymorphisms with risk of SCCOP recurrence, using hazard ratios (HRs) and their 95% confidence intervals (CIs) among both overall patients and patients with HPV16 (+) tumors. While because of the relatively small sample size and the few recurrence events of SCCOP patients and patients with HPV16 (+) tumors, we did not include similar analyses for patients with HPV16 (-) tumors in this study. Statistical analyses were performed with SAS software (version 9.2; SAS Institute Inc., Cary, NC). All tests were two-sided, and \( P < .05 \) was considered significant.

**Results**

**Demographics and Risk Factors for the Study Population**

We had a total of 1226 SCCOP patients from May 1995 through October 2013, and 218 patients were excluded from the final analysis due to insufficient information available about follow-up and treatment or no blood samples available for genotyping. Therefore, our final analysis included 1008 incident patients with SCCOP. The overall recurrence rates of SCCOP, the 5-yr recurrence rate, and the associations between DFS and patient’s characteristics including age at diagnosis, sex, ethnicity, smoking, alcohol use, comorbidity group, index cancer stage, and treatment, were previously reported [18]. The median follow-up time and mean age at diagnosis among all patients, patients with SCCOP recurrence, and patients without recurrence were also previously reported [18]. As shown in Table 1, the Kaplan–Meier univariate analysis showed that the 5-yr rate of recurrence was significantly different between two groups categorized by age at diagnosis, ethnicity, smoking status, alcohol drinking, comorbidity group, and treatment (all \( P < .05 \)), while no significant difference was found between the groups for sex and index cancer stage (both \( P > .05 \)).

**Joint Effect of E2F1-rs3213180 Polymorphism and HPV-16 Serology on the DFS of SCCOP**

SCCOP patients with the common homozygous E2F1-rs3213180 GC/CC genotypes had significantly better DFS than those with GG genotype (log-rank, \( P < .0001 \); Figure 1). Furthermore, among 324 patients with HPV16 (+) SCCOP only, we found that the DFS was significantly better in patients carrying the E2F1-rs3213180 GC/CC genotypes than in those carrying the corresponding common homozygous GG genotype (log-rank, \( P < .001 \); Figure 1).

**Joint Effect of E2F1-rs3213180 Polymorphism and HPV-16 Serology on the Recurrence Risk of SCCOP**

We further performed multivariable Cox proportional hazards regression analyses to assess the associations of E2F1-rs3213180 polymorphism with recurrence risk in patients with SCCOP. As shown in Table 2, compared with patients with the E2F1-rs3213180 GG common homozygous genotype, patients with the E2F1-rs3213180 GC/CC genotypes had significantly lower risk of SCCOP recurrence (HR, 0.2, 95% CI, 0.1–0.4). Given the important role of HPV and E2F1 in the E7-RB-E2F pathway which is involved in regulation of apoptosis and proliferation, cell-cycle progression, and tumorigenesis, we further evaluated the associations of this polymorphism with risk of recurrence among the patients with HPV16 (+) SCCOP. As shown in Table 3, among HPV16 (+) patients, the E2F1-rs3213180 GC/CC variant genotype had significantly lower risk of recurrence than the E2F1-rs3213180 GG common homozygous genotype (HR, 0.2, 95% CI, 0.1–0.4).

**Discussion**

In this large cohort study of 1008 patients with incident SCCOP, we found that the E2F1-rs3213180 variant was significantly associated with the risk of SCCOP recurrence, especially in patients with HPV16 (+) tumors. The results suggested that E2F1-rs3213180 may predict the recurrence risk of SCCOP, particularly for those with HPV16 (+) tumors. The exact mechanism by which this E2F1 gene variant predicts the recurrence risk of SCCOP remains unclear, while it is likely that genetic variants within the functional regions of E2F1 may result in the altered expression of this gene and thus affect susceptibility of response to treatment such as radiotherapy, thus leading to different

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**Table 1. Characteristics of Patients With SCCOP (N = 1008)**

| Variable                  | No. (%) of Patients | No. of Patients With Recurrence | 5-year Recurrence Rate(%) | \( P^* \) |
|---------------------------|---------------------|---------------------------------|---------------------------|---------|
| No. of patients           | 1008 (100)          | 181                             | 0.20                      |         |
| Age                       |                     |                                 |                           |         |
| ≤57 years                 | 621 (61.6)          | 85                              | 0.15                      | <.0001  |
| >57 years                 | 387 (38.4)          | 96                              | 0.27                      | .0030   |
| Sex                       |                     |                                 |                           |         |
| Male                      | 872 (86.5)          | 161                             | 0.20                      | .3110   |
| Female                    | 136 (13.5)          | 20                              | 0.19                      | .9220   |
| Ethnicity                 |                     |                                 |                           |         |
| Non-Hispanic white        | 913 (90.6)          | 146                             | 0.17                      | <.0001  |
| Other                     | 95 (9.4)            | 35                              | 0.41                      | .2380   |
| Smoking                   |                     |                                 |                           |         |
| Never                     | 388 (38.5)          | 51                              | 0.14                      | .0004   |
| Ever                      | 620 (61.5)          | 130                             | 0.23                      | .0030   |
| Alcohol drinking          |                     |                                 |                           |         |
| Never                     | 761 (75.5)          | 155                             | 0.25                      | .0005   |
| Ever                      | 247 (24.5)          | 26                              | 0.10                      | .3920   |
| Comorbidity               |                     |                                 |                           |         |
| None or mild              | 913 (90.6)          | 157                             | 0.19                      | .0370   |
| Moderate to severe        | 95 (9.4)            | 24                              | 0.27                      | .2080   |
| Index cancer stage        |                     |                                 |                           |         |
| 1 or 2                    | 72 (7.1)            | 11                              | 0.19                      | .5280   |
| 3 or 4                    | 936 (92.9)          | 170                             | 0.20                      | .0270   |
| Treatment                 |                     |                                 |                           |         |
| X/XC/XS/XS                | 947 (93.9)          | 166                             | 0.19                      | .0030   |
| XSC                       | 61 (6.1)            | 15                              | 0.32                      | .0030   |

\( P^* \): Log-rank test for DFS between the two groups. X, radiotherapy; C, chemotherapy; S, surgery.
clinical prognosis. Therefore, E2F1 polymorphisms could be served as potential predictive biomarkers of recurrence in SCCOP patients, and it can help surgeons make individualize treatment which lead to an improved prognosis and better quality life for SCCOP patients.

The E7 gene of HPV can constitute complex formation with pRB1 in Rb gene families which have negative control function to the cell growth, and release E2F1 transcription factors in the cells. Free E2F1 can activate the expression of some host genes in the process of cell cycle; inactivate the corresponding proteins of p16 and Rb [20]. Thus, it is biologically plausible that genetic variants within the 3'-UTR of E2F1 might affect expression of E2F1 protein which, in turn, could affect p16 expression level, and thus affect susceptibility of response to radiotherapy. Although the functional relationship of the E2F1-rs3213180 polymorphism has not been clarified yet, our association results can partially support a functional correlation between this polymorphism and E2F1 function, which may provide preliminary evidence of biological plausibility. Thus, the rs3213180 polymorphism may cause significant changes in the expression levels of E2F1, which result in the alternation of cell cycle progression and induction of p16 expression. Therefore, it is likely that this E2F1 polymorphism may have potentially functional effect on p16 expression, thus leading to different recurrence risk. However, these findings should be investigated in future studies.

In recent years, growing evidence suggests that miRNAs directly binds to the sites in its 3′UTR and promoter of the E2F1, activating its transcription, which in turn modulates the translation of E2F1 mRNAs [21]. The altered E2F1 expression may affect the expression of genes involved in DNA repair, cell cycle progression, and apoptosis [8]. Zhao et al. [16] observed that E2F1-rs3213180 had a significant association with E2F1 expression, implying that this variant could contribute in part to E2F1 post-transcriptional regulation. Gorgoulis et al. [22] reported that increased E2F1 positivity demonstrated a significant increase in their growth indexes and were associated with adverse prognosis in non-small cell lung carcinomas. Similarly, Gao, et al. [23] also reported that E2F1 overexpression were significantly associated with tumor stage and poor prognosis in non-small cell lung carcinomas. Liang et al. [24] demonstrated that E2F1 might function as a biomarker that can differentiate patients with recurrent and non-recurrent disease following radical prostatectomy, while no studies have investigated the modifying effect of E2F1 polymorphisms on prognosis of SCCOP patients, especially for HPV-associated SCCOP. In our previous study, we found that certain selected SNPs within the E2F1 genes had a combined effect on the risk of SCCN in a genotype dose–response manner [25]. We also found that polymorphism at the 3′UTR miRNA binding site of E2F1-rs3213180 was associated with risk of HPV-associated OSCC, particularly for SCCOP [17]. In our current study, we found a significant association between the E2F1-rs3213180 variant genotypes and an increased risk of SCCOP recurrence. Although these studies have demonstrated the significant associations of E2F1 polymorphisms with cancer risk and prognosis, we can’t exclude the effects of other factors, such as genetic background, cancer type, environmental factors, tumor size, tumor stage, treatment, adequacy of adjustment for other confounding factors, and different study populations [26].

Except for the factors such as clinical, genetic, and lifestyle factors, affected risk of patients with SCCOP, tumor HPV status also influenced the risk of SCCOP recurrence. Patients with HPV16 (+) SCCOP had a better prognosis than those with HPV (-) SCCOP. Our previous study had found that E2F1 was associated with risk of HPV-associated SCCOP [17]. We further explored the role of this E2F1 polymorphism in recurrence risk in SCCOP patients who were stratified by tumor HPV16 status. We found that among patients with HPV16(+) tumors, the patients with variant genotypes of the E2F1 polymorphism had better DFS and lower recurrence risk than those with homozygous genotype of this polymorphism, suggesting that E2F1-rs3213180 variant genotypes interact with HPV status partially via the E7-RB-E2F1 pathway to affect SCCOP recurrence risk. Although the molecular mechanisms underlying this finding in patients with HPV16(+) SCCOP remain incompletely understood, the variant genotypes of the polymorphism may affect...
DNA repair capacity or apoptosis through controlling the cell cycle [27], which supports the notion that SCCOP is mainly driven by HPV [28,29]. Thus, E2F1 variants may affect susceptibility to radiotherapy and lead to different recurrence outcomes. However, this hypothesis should be investigated in future studies.

Our study reveals several strengths including its relatively large sample size, inclusion of tumor HPV status, and analysis of a homogeneous tumor site in SCCOP, while there are also several limitations inherited in this study including the lack of details on radiotherapy (e.g., dosage, treatment cycles, duration, etc.), the limited number of outcome events in HPV16 (+), and our cases recruited at a single cancer center with a majority of racial and ethnic group of non-Hispanic white. Taken together, our findings support a significant role of a functional variant of E2F1-rs3213180 at the miRNA binding site in E2F1 gene in susceptibility to disease recurrence of SCCOP, especially in patients with HPV16 (+) tumors. However, large and prospective studies are needed to validate our results and further explore the molecular mechanisms underlying the observed associations.

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Conflict of Interest
The authors declare no conflicts of interest.

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