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Alterations in *RD^{INK4/ARF}*-mediated *en bloc* regulation of the *INK4-ARF* locus in human squamous cell carcinoma of the head and neck

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Abstract

The presence of *RD^{INK4/ARF}* (*RD*) enhancer in the *INK4-ARF* locus provides a novel mechanism to simultaneously increase the transcription of *p15^{INK4b}* (*p15*), *p14^{ARF}* (*p14*), and *p16^{INK4a}* (*p16*). While such up-regulation can be repressed through interactions between *RD* and oncoproteins CDC6 and BMI1, little is known about the involvement of *RD* in cancer. In this study we investigated *RD* deletions in 30 squamous cell carcinoma of the head and neck (SCCHN) and the patient-matched High At-Risk Mucosa specimens (HARM, “phenotypically normal” tissues neighboring SCCHN foci but beyond the surgical resection margin). *RD* was deleted (homozygously/heterozygously) in SCCHN and HARM at the incidence of 36.7% (11/30) and 13.3% (4/30), respectively. In comparison, no *RD* deletion was detected in 26 oral buccal brush biopsy specimens from healthy donors. Both *p16* and *p14* were lowly expressed in SCCHN and HARM, and their mRNA expression levels were positively associated with each other ($p < 0.01$). Moreover, *BMI1* was highly expressed in both SCCHN and HARM, and *BMI1* over-expression was associated with *p16* down-regulation in SCCHN ($p < 0.05$). These results indicate that *RD* deletion and *BMI1* overexpression frequently occur in the early stage of oral carcinogenesis and *BMI1* overexpression may down-regulate the transcription of *p16* and *p14* through interfering with *RD*.

Keywords

RD enhancer; the *INK4-ARF* locus; BMI1; genetic deletion; *en bloc* regulation

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INTRODUCTION

The *INK4-ARF* locus encodes three linked tumor suppressors, P16^{INK4A} (P16, *CDKN2A*), P14^{ARF} (P14, *CDKN2A*, alternate reading frame), and P15^{INK4B} (P15, *CDKN2B*), and is involved in the predominant tumor suppressor pRb and P53 pathways, known to be deregulated in essentially all human cancers [1–4]. These three genes are driven by their respective promoters and have distinct open reading frames (Figure 1A). P16 and P15 function as specific cyclin-dependent kinase (CDK) 4/CDK6 inhibitors, and negatively modulate the phosphorylation of pRb and the subsequent E2F-mediated transactivation of genes required for entry into the S phase, thus inactivation of *p16* and/or *p15* allows cells to escape cell cycle arrest in G₁. P14 binds to HDM2 (MDM2 in mice), a protein with the E3 ubiquitin ligase activity, and promotes the latter's degradation, resulting in P53 activation and G₁ and G₂ arrest. Interestingly, it has been reported recently that there exists a putative replication origin in 1.5 kb upstream of the ATG start codon of the *p15* gene (Figure 1), and the location of this replication origin coincides with a DNA element of about 350 bp conserved among mammalian *INK4-ARF* loci [5,6]. Further studies demonstrated that this regulatory domain, *RD*^{*INK4/ARF*} (hereafter, *RD*), is a relevant transcriptional regulatory sequence, and it enhances the expression of *p16*, *p15*, and *p14*^{ARF}, i.e. *RD* exhibits a global positive effect on the transcription of the entire *INK4-ARF* locus [5]. Moreover, it has been demonstrated that the transcription of the entire *INK4-ARF* locus can be repressed upon binding of CDC6 and BMI1 to *RD* [6,7]. Of note, CDC6 is an essential DNA replication modulator [8,9], and BMI1 is a Polycomb (PcG) protein that functions to generate and recognize histone modifications in genomic domains with clusters of genes [10,11]. Accordingly, high levels of CDC6 and BMI1 bring about recruitment of histone deacetylases and heterochromatinization of the *INK4-ARF* locus as well as a concomitant silencing of all three genes encoded by this locus in a *RD*-dependent manner [5,7]. Apparently, such *RD*-mediated *en bloc* regulation provides a novel mechanism to modulate P15, P14, and P16 at the transcription level, and perturbations to this mechanism, such as genetic alterations of *RD* and aberrant expression of *CDC6* and *BMI1* potentially impair both pRb and P53 pathways, thus contributing to cancer progression. This notion is further supported by the following observations. First, CDC6 and BMI1 have been found to be frequently over-expressed in a variety of human cancers, such as non-small cell lung cancers (NSCLC), brain cancers, pancreatic adenocarcinomas, and squamous cell carcinoma of the head and neck (SCCHN) [8–11]. Secondly, a recent study in our laboratory has shown that *RD* is deleted (homozygously or heterozygously) in human pancreatic and oral cancer cells with a considerably high incidence, and such deletion event is not associated with the well-known deletions of *p16* or *p14* in cancer cells [12]. However, while genetic alterations of *p16* and *p14* (as well as *p15*) in human cancers have been extensively investigated [1–4], little is known about the prevalence of *RD* alteration and its potential contribution to carcinogenesis.

Here we report the incidence of *RD* deletion (homozygous and heterozygous) in a cohort of 30 SCCHN patients. Out of 30 SCCHN specimens, three and eight specimens harbored homozygous and heterozygous deletions of *RD*, respectively. In the patient-matched, “phenotypically normal,” High At-Risk Mucosa tissues (HARM), *RD* was homozygously

deleted in one specimen and heterozygously deleted in three specimens. In contrast, no *RD* deletion was found in 26 oral brush biopsy specimens from healthy donors. We also demonstrated that *BMII* was highly expressed at the mRNA level in both SCCHN and HARM specimens, and such up-regulation is associated with concomitant down-regulation of the transcription of *p14* and *p16* in these tissues. These findings support our hypothesis that perturbing *RD*-mediated *en bloc* regulation through *RD* deletion and aberrant expression of *BMII* frequently occurs at the early, “initiating” stage of SCCHN, and such events could contribute considerably to oral cancer risk progression through down-regulating both *p14* and *p16*.

MATERIALS AND METHODS

Procurement of human oral tissue samples

All 30 patient-matched SCCHN and HARM tissues were obtained at the time of standard-of-care surgical resection at The Ohio State University Wexner Medical Center, Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, following Institutional Review Board approved protocols. After surgical resection, portions of the tissues were used for DNA and RNA extraction as previously described [13,14]. Additional oral brush biopsy specimens from healthy volunteers were collected using an OralCDx brush (Oral Cancer Prevention International; Suffern, NY) and used for DNA and RNA isolation. While genomic DNAs of high quality were purified from all 26 buccal brush specimens, RNAs of good quality were obtained from only 16 of these specimens, which were subsequently used for mRNA expression analysis.

Detection of genomic deletions in *RD* and *INK4-ARF* in human oral tissues

Homozygous and heterozygous deletions of *RD* were determined using multiplex qPCR as previously described [12,13]. The primers and probes for *RD* and β -actin (*ACTB*, endogenous reference) are listed in Supplementary Table 1. Briefly, genomic DNA admixes containing TE-1177 oral epithelial cell DNA with an intact *INK4-ARF* locus [12] and Panc-1 cell DNA harboring a homozygous deletion of the entire *INK4-ARF* locus were combined at various ratios (100:0, 75:25, 50:50, 25:75, 0:100) and analyzed using normalized quantification cycle (Cq) values as follows:

$$\Delta\Delta Cq = (Cq_{RD}^{Sample} - Cq_{RD}^{TE-1177}) - (Cq_{ACTB}^{Sample} - Cq_{ACTB}^{TE-1177})$$

The Cq values were then plotted against known ratios of *RD* (in exponential form) to obtain a calibration line with a correlation coefficient of -0.99 (Figure 1B), demonstrating that the dosage of *RD* in a sample can be accurately approximated using this technique [13]. Subsequently, the gene dosage of *RD* in tumors was determined and the results were interpreted using the following percentiles: (i) dosage $<25\%$ indicates homozygous deletion (*RD* $-/-$), (ii) dosage between 25% – 75% indicates hemizygous deletion (*RD* $+/-$), and (iii) dosage $>75\%$ indicates *RD* wild type (*RD* $+/+$) [13]. Deletions of *p15*, *p14*, and *p16* were determined through similar qPCR-based approaches as previously demonstrated [12]. All experiments were performed in triplicate.

Detection of human papillomavirus 16 (HPV16) E6 gene in human oral tissues

Since HPV16 is the major human papillomavirus subtype that infects oral tissues [15], a PCR-based presence/absence assay was performed as previously described to detect the HPV16 E6 gene in oral tissue DNA samples [16]. Briefly, endpoint PCR assays were performed for 30 cycles (denaturation, 30 seconds at 94 °C; annealing, 30 seconds at 60 °C; extension, 30 seconds at 72 °C) using primers specific for HPV16 E6: forward, 5'-TCAAAAGCCACTGTGTCCTG-3'; reverse, 5'-CGTGTCTTGTGATCTGCA-3'. The 120 bp PCR product was subjected to 10% PAGE electrophoresis and visualized using ethidium bromide staining and UV transillumination.

Quantitative determination of *p16*, *p14^{ARF}*, *p15*, *p18^{INK4c}* (*p18*), *CDC6*, and *BM11* transcriptional expression

Extraction of total RNA and first-strand cDNA synthesis were performed as previously described [14]. Quantitative RT-qPCR reactions were carried out using a StepOne Real-Time PCR System (Life Technologies/Applied Biosystems) and pre-validated TaqMan gene expression assays as listed in Supplementary Table 1. Human hypoxanthine phosphoribosyltransferase (*HPRT1*) was used as an endogenous reference for normalized gene expression [12]. Each gene was amplified separately, and all experiments were performed in triplicate. The relative gene expression level of a target was determined using a comparative Cq method [17], in which data for a target gene were normalized to that for *HPRT1*, the reference gene, such that: $Cq = Cq_{target} - Cq_{HPRT1}$, relative gene expression = 2^{-Cq} .

Statistical analyses

All data in this study were analyzed using the R software package (version 2.15.0, <http://www.r-project.org>). Fisher's exact tests and Pearson's χ^2 tests were used for deletion (count) data. Welch two-sample *t*-tests were used for comparisons between SCCHN tumor and Control brush biopsy gene expression data, and between HARM and Control brush biopsy gene expression data. Paired *t*-tests were used for comparisons between SCCHN tumor and HARM gene expression data. Pearson's product-moment correlation tests were applied for the correlation(s) between different genes in mRNA expression. All tests are two-sided and the significance level was at $\alpha=0.05$.

RESULTS

Patient clinico-pathologic features

Thirty SCCHN patients and 26 healthy volunteers were included in the current study. Demographics and clinico-pathologic features of these cancer patients and healthy controls are summarized in Table 1. There are no significant differences apparent between the two populations with respect to sex, race, and ethnicity. However, there are more men than women in the SCCHN patient group as well as in the healthy control group. In each case, participants were acquired as part of an enrollment process without gender or race restrictions. The majority of male patients in the SCCHN study is consistent with the observation that men are more likely to be diagnosed with SCCHN [18]. Additionally, there

are more white participants than people from other races in both groups. As noted, participants were eligible and acquired without race restrictions. However, these two groups do demonstrate a significant difference in age ($p < 0.01$) at the time of tissue collection. Specifically, while 16/26 healthy volunteers were under at the age of 45, most of the SCCHN patients (24/30) were 45 or older. Indeed, it is well documented that most of SCCHN patients are between 50–70 years old, and this studies age distribution is likely a reflection of typical SCCHN patient characteristics [18].

Tobacco use (typically cigarette use), alcohol consumption, and human papillomavirus (HPV) infection are three primary risk factors of SCCHN [15,19–21]. As shown in Table 1, the SCCHN group and the healthy volunteer group are significantly different from each other ($p < 0.05$) with respect to smoking status and HPV16 infection. 70% of SCCHN patients (21/30) were ever-smokers (current or former) and 16.6% of SCCHN specimens (5/30) demonstrated evidence of HPV16 infection. In comparison, 34.6% of the healthy volunteers (9/26) were ever-smokers and no evidence of HPV16 infection was detected in any of these control tissues. As for alcohol consumption, current or former drinkers covered the majorities of both the SCCHN (76.7%, 23/30) and healthy volunteer groups (69.3%, 18/26).

Within the SCCHN group, no significant association was found between any two of the following features: age, tumor location, tumor stage, smoking status, alcohol use, and HPV16 infection. Importantly, it is essential to recognize that the power of these statistical analyses could be compromised due to the small sample size after stratification.

***RD* deletions in human oral tissues**

RD is an A/T-rich DNA segment and it contains no canonical CpG island motifs. To date, deletion (homozygous and heterozygous) is the only general molecular mechanism we have found to inactivate *RD* in cancer cells [12]. Hence, we first investigated potential *RD* deletions in oral specimens using qPCR-based deletion assays as previously described [12]. The genetic status of *RD* in these oral specimens is listed in Table 2. In the SCCHN group, three and eight specimens harbored homozygous and heterozygous deletions, respectively. In the patient-matched HARM group, four *RD* deletion events, including one homozygous deletion and three heterozygous deletions, were identified. In striking contrast, no *RD* deletion was detected in the oral buccal specimens obtained from healthy volunteers. Consequently, the incidence of *RD* deletion in SCCHN (36.7%, 11/30) is significantly higher than that observed in the healthy volunteer group (0/30) ($p < 0.01$) (Table 3). Further studies showed that *RD* deletion in SCCHN was non-significantly associated with age, tumor location, tumor stage, smoking status, alcohol use, or HPV16 infection. It is also worthwhile to note that for the four HARM specimens harboring *RD* deletions (21H1, 25H1, 28H1, and 30H1), *RD* deletions were detected in their patient-matched SCCHN samples (21T1, 25T1, 28T1, and 30T1). Interestingly, while *RD* was homozygously deletion in 21H1, it demonstrated a heterozygous deletion in its patient-matched tumor (21T1). Oral carcinogenesis is a multistep process characterized by a microenvironment of “field effect” damages from common carcinogenic exposures, including alcohol and tobacco use. The unexpected observation of a complete *RD* deletion in what is posited to represent a less

malignant phenotype (HARM) compared to a patient-matched malignant tumor phenotype (SCCHN) may be a reflection of this field cancerization effect. In other words, there is no additional evidence at this time to definitively establish a clonal derivation of 21T1 from 21N1. Furthermore, because the SCCHN tissues were isolated as intact tumor tissues (i.e. not microdissected), the differences between the tissue *RD* profiles may reflect the heterogeneity of some solid tumors in this case.

We also investigated deletions of *p14*, *p15*, and *p16* in these oral tissue samples. As listed in Table 2, the incidences of *p14*, *p15*, and *p16* deletions in the SCCHN group are 43.3% (13/30), 23.3% (7/30), and 23.3% (7/30), respectively. In the HARM group, the corresponding incidences are 3.3% (1/30), 10% (3/30), and 10% (3/30), respectively, whereas no deletion in any of these genes was identified in the healthy volunteer group. For each of these tumor suppressor genes, their incidences of genetic deletion are significantly higher in the SCCHN group ($p < 0.05$) compared with the healthy volunteer group. As observed for *RD* deletion status, the deletion status of *p14*, *p15*, or *p16* is non-significantly associated with age, tumor location, tumor stage, smoking status, alcohol use, and HPV16 infection.

Interestingly, the composite incidences of any deletion within the *INK4-ARF* locus, i.e. any *RD*, *p14*, *p15*, *p14*, or *p16* deletion, in SCCHN and HARM are 56.7% (17/30) and 23.3% (7/30), respectively, both of which are significantly high compared with the 0% incidence in the healthy volunteer group ($p < 0.05$). These results suggest that the *INK4-ARF* locus is indeed vulnerable to deletion in the progressive development of SCCHN. Moreover, there is a strong association between *RD* deletion and *p15* deletion in SCCHN ($p < 0.01$). Such association could be attributed in part to the physical genomic proximity between *RD* and *CDKN2B* (about 1.5 kb). However, these two deletion events appear to be unlinked and independent from each other in specific samples. While many SCCHN specimens harbored both *RD* and *p15* deletions, other samples demonstrated only *RD* or *p15* deletions. For example, 04T1, 06T1, and 19T1 demonstrate an intact *RD* region (*RD*+/+) but harbored *p15* deletions. In contrast, while an *RD* deletion was found in 21T1, the remaining *INK4-ARF* locus remained intact (*p14*+/+, *p15*+/+, *p16*+/+). Similarly, *p15* deletion is positively associated with *p16* deletion in SCCHN ($p < 0.01$). However, in contrast to the possible contribution of genomic proximity for *RD* and *p15*, such an association is less ascribable to genomic nearness, since there is no association between *p15* and *p14* ($p = 0.67$) or *p14* and *p16* ($p = 0.30$) even though *p14* is physically located between *p15* and *p16* genomic positions. Additionally, *RD* deletion appears to be poorly associated with *p14* deletion ($p = 0.47$) or *p16* deletion ($p = 0.37$) in SCCHN. Nonetheless, this apparent “dissociation” does not rule out the possibility that a single molecular event might lead to the truncation of all of *RD*, *p15*, *p14*, and *p16* or several of them. For example, a single deletion event could eliminate the entire *INK4-ARF* locus resulting in the heterozygous deletion status for each of *RD*, *p14*, *p15*, and *p16* in 13T1 and 30T1. Moreover, in 29T1 *RD*, *p15*, and *p14* were heterozygously deleted while *p16* remained intact, suggesting that a locus deletion event occurred prior to the *p16* region of the *INK4-ARF* locus. Taken together, our results reveal that in spite of their intrinsic genomic proximity, deletions of *RD*, *p14*, *p15*, and *p16* are

often independent of each other. From this perspective, frequent *RD* deletions in SCCHN should be regarded as a “novel” mechanism to inactivate the *INK4-ARF* locus.

Up-regulation of *BM11* in oral tissues

Subsequently, we evaluated the mRNA expression of *CDC6* and *BM11* in these oral tissues using pre-validated TaqMan gene expression assays [12]. As shown in Figure 2A, *CDC6* was significantly down-regulated in HARM ($p < 0.01$) but not in SCCHN ($p > 0.10$) with respect to the healthy volunteer group. These findings are interesting, since they are counter to our original hypothesis that *CDC6* would be up-regulated in SCCHN. Intriguingly, *BM11* was significantly up-regulated in both HARM ($p < 0.01$) and SCCHN ($p < 0.01$) (Figure 2B). Since there is no significant difference in *BM11* mRNA expression between the SCCHN and HARM groups, up-regulation of *BM11* may represent a molecular event occurring during the early stage of oral carcinogenesis. More interestingly, there exists a strong association between the *BM11* mRNA expression and the genetic status of *RD* in SCCHN ($p < 0.01$) (Figure 2C). *BM11* mRNA expression in 19 SCCHN tissues with intact *RD* (*RD*+/-) was significantly higher than that in 11 SCCHN tissues containing homozygous or heterozygous deletions of *RD* (*RD*+/-, *RD*-/-), while there is no significant difference in *BM11* mRNA expression between SCCHN with homozygous *RD* deletions (*RD*-/-, $n=3$) and SCCHN with heterozygous *RD* deletion (*RD*+/-, $n=8$). In comparison, no significant difference is observed in *BM11* mRNA expression between HARM tissues with intact *RD* ($n=26$) and HARM tissues with *RD* deletions ($n=4$). These results suggest that a feedback mechanism could exist between up-regulation of *BM11* and *RD* deletion in SCCHN. In other words, the “intactness” of *RD* induces over-expression of *BM11*, thus repressing the enhancing impact of *RD* on the transcriptional regulation of the *INK4-ARF* locus. We also stratified the SCCHN group with regard to age, tumor location, tumor stage, smoking status, alcohol use, and HPV16 infection, and compared them with *BM11* mRNA expression. Our results do not support any significant association between *BM11* mRNA expression and any of the interrogated variables (data not shown).

Concomitant down-regulation of *p14* and *p16* in SCCHN

To explore the potential impact of *BM11* up-regulation on the transcription of the *INK4-ARF* locus, we continued to estimate the mRNA expression of *p14*, *p15*, and *p16* in these oral tissues. As shown in Figure 3A, *p16* was significantly down-regulated at the mRNA level in *p16*+/- HARM tissues retaining intact *p16*, in comparison with the healthy volunteer group ($p < 0.01$). Such down-regulation of *p16* at the mRNA level became more dramatic in *p16*+/- SCCHN tissues. The mRNA expression of *p16* in *p16*+/- SCCHN was even lower than that in *p16*+/- HARM tissues ($p < 0.05$). Similarly, the mRNA expression of *p14* was down-regulated in both *p14*+/- SCCHN tissues ($p < 0.01$) and HARM *p14*+/- HARM samples ($p < 0.01$) (Figure 3B). However, there is no significant difference in the mRNA level of *p14* between *p14*+/- SCCHN and *p14*+/- HARM groups. We also did not observe any significant association between the mRNA expression of *p14* or *p16* and the clinical variables of age, tumor location, tumor stage, smoking status, alcohol use, or HPV16 infection. The mRNA expression level of *p15* was near the limits of meaningful detection (i.e. Cq values approaching negative control “blank” values) in all tested oral specimens

despite the use of pre-validated TaqMan gene expression assays. Consequently, comparisons and correlations involving *p15* mRNA levels between different groups were not pursued in this study.

We did examine the potential association between *p14* and *p16* expression in these oral tissue samples. Our results demonstrated that in 19 *p14*^{+/+} and *p16*^{+/+} SCCHN, the Pearson correlation coefficient between the mRNA levels of *p14* and *p16* was 0.91 ($p < 0.01$). The corresponding value in 26 *p14*^{+/+} and *p16*^{+/+} HARM tissues was 0.97 ($p < 0.01$). These findings support a strong positive association between *p14* and *p16* mRNA expression levels in SCCHN and HARM. In marked contrast, the association between *p14* and *p16* mRNA expression levels in the healthy volunteer group ($n = 16$) was poor ($r = 0.42$, $p = 0.15$). Furthermore, up-regulation of *BM11* and down-regulation of *p14* or *p16* was generally negatively associated in SCCHN tissues, with a Pearson correlation coefficient between *BM11* and *p14* mRNA levels in 23 SCCHN of -0.35 ($p = 0.10$), and the corresponding value between *BM11* and *p16* of -0.40 ($p = 0.07$).

Overall, *p14* and *p16* tended to be concomitantly down-regulated in HARM and SCCHN, and such down-regulation appeared to be associated with up-regulation of *BM11* in these oral tissues. Surprisingly, the level of *p16* mRNA in SCCHN harboring both intact *p16* and *RD* (*p16*^{+/+}, *RD*^{+/+}; $n = 16$) is comparable to that in SCCHN with intact *p16* but deleted *RD* ($n = 7$, including 1 *RD*^{-/-} and 6 *RD*^{-/+}; data not shown), suggesting that *RD* deletion in these SCCHN samples does significantly impact *p16* transcription. Arguably, these results appear to not support the role of *RD* as a locus transcription enhancer. However, as demonstrated earlier (Figure 2C), the expression level of *BM11* mRNA in *RD*^{+/+} SCCHN was significantly elevated compared with *RD*^{+/-} or *RD*^{-/-} SCCHN. We hypothesize that the enhancing effect of *RD* on the transcription of *p16* is countered by the repressing impact of highly expressed *BM11* in *RD*^{+/+} SCCHN. This intact *RD* genotype leads to a *p16* mRNA expression level comparable to that in *RD* deleted tissues. From this perspective, the coordination between *RD* and *BM11*, rather than *RD* or *BM11* independently, may impact the transcription of *p16*, as well as *p14* and *p15* the majority of SCCHN.

DISCUSSION

The *INK4-ARF* locus plays a central role in tumor suppression as reflected by the observation that nearly 50% of all human cancers harbor an inactivated *INK4-ARF* locus [1–4], and that SCCHN demonstrate a 40–60% inactivation frequency [22]. While deletions, methylation, and point mutations in each individual *INK4-ARF* gene (*p14* or *p15* or *p16*) are well-known inactivating mechanisms [3], our current studies demonstrate that disrupting *RD*-mediated *en bloc* regulation may represent a novel mechanism to functionally inactivate the entire *INK4-ARF* locus. On one hand, *RD* is frequently deleted in malignant SCCHN tumors and “field defective” non-tumor HARM in combination with or independent of deletions of individual *INK4-ARF* gene(s). On the other hand, *BM11* is up-regulated at the mRNA level in both SCCHN and HARM, which tends to be associated with the concomitant down-regulation of *p14* and *p16* in these oral tissues. In addition, the mRNA level of *BM11* in *RD*^{+/+} SCCHN is significantly higher than that in SCCHN with *RD* deletions (*RD*^{+/-} or *RD*^{-/-}), indicating that the enhancing effect of *RD* on the transcription

of the *INK4-ARF* locus is functionally counteracted by elevated expression of *BMII* through currently unknown mechanisms. As addressed below, these results provide fundamental advances to the understanding of the contributions of the tumor suppressive *INK4-ARF* locus in oral carcinogenesis.

First, *RD/BMII*-mediated deregulation of the *INK4-ARF* region has a global effect on all three tumor suppressors encoded by the *INK4-ARF* locus [5,7,23], thus providing an inclusive effect greater than the individual genetic inactivation events of the *p16* and/or *p14* genes. While many genetic alterations in the *INK4-ARF* locus interrupt either the pRb pathway or the P53 pathway, perturbations in *RD/BMII* regulation can significantly impair both tumor suppressive pathways. Furthermore, functional redundancy allows P15 and P18^{INK4C} (P18, another INK4 protein homologous to P16) to successfully substitute for P16 in tumor suppression in the absence of *p16* [24,25]. In most of human cancers the *p15* gene remains intact while genetic alterations occur at the *p16* (and/or *p14*) gene, thus the aforementioned redundancy between P15 and P16 may compromise the significance of *p16* inactivation in cancer development. However, perturbations in *RD/BMII* regulation will abolish such functional redundancy between P15 and P16. Furthermore, it has been reported that *BMII* is able to down-regulate P18 functioning in cells through mechanisms yet to be defined [10,26]. Hence, elevated expression of *BMII* in SCCHN and HARM may also negatively impact P18 and as its role in tumor suppression. Indeed, as shown in Figure 3C, *p18* was significantly down-regulated at the mRNA level in SCCHN ($p < 0.01$) in comparison with the HARM group or the healthy volunteer group, and such down-regulation was strongly associated with up-regulation of *BMII* in SCCHN with a correlation coefficient of -0.65 ($p < 0.01$; $n = 30$).

Second, even though methylation and point mutations of individual *INK4-ARF* genes were not investigated in our current study, previous studies have shown that genetic inactivation of *p16* or *p14* occurs in SCCHN at considerably high frequencies [23,27,28]. In regard to this, the functions of P16 (or P14, or P15) in SCCHN are influenced by the cumulative (i) overall global effect of aberrant *RD/BMII* interaction, and (ii) specific local effect of individual gene alterations. Consequently, the physiological up-regulation or down-regulation of P16 in SCCHN depends on the coordination and summation of these distinct regulatory events. For example, the frequently observed over-expression of *p16* (both wild-type and mutant) in SCCHN cannot be ascribed to genetic inactivation of the *p16* gene [29]. However, alterations that appropriately affect *RD/BMII* interaction, such as amplification of *RD* or down-regulation of *BMII*, do have the potential to up-regulate *p16*.

Third, over-expression of *BMII* and its negative association with *p16* have been found in many cancers and cancer stem cell lines [10,11,23], but the underlying mechanisms remain to be further elucidated. We propose that the *RD/BMII* interaction could be one of these mechanisms. Unlike permanent gene-specific *INK4* alterations such as mutation and deletion, the repression of *BMII* on the transcription of the *INK4-ARF* locus is reversible and may be susceptible to post-translational modifications (most likely, phosphorylation) and protein/protein interactions [30]. Hence, changes in *BMII* transcriptional expression or protein modification could be especially sensitive to certain oncogenic stresses and occur prior to DNA damage, consequently providing a valuable biomarker for early oral cancer

detection and intervention, as suggested by the elevated expression of *BM11* in non-tumor, phenotypically “normal” HARM tissues residing in the oral field cancerization microenvironment (Figure 2B).

Last, it has been reported that *CDC6* is involved in *BM11*-mediated repression of the *INK4-ARF* locus through physical interactions [7], however, there is no association observed between the transcriptional expression levels of *CDC6* and *BM11* in these SCCHN and HARM tissues. As shown in Figure 2, *BM11* was over-expressed in both SCCHN and HARM in comparison with the healthy control specimens, without any significant up-regulation of *CDC6*. While further molecular and cancer biology studies are needed to clarify the coordination or dissociation between *CDC6* and *BM11*, it is important to note that such “coordination” is based on protein-protein interactions between *CDC6* and *BM11*. Hence, the kinetic and dynamic nature of *CDC6*-*BM11* binding as well as other biophysical/biochemical properties, rather than the mRNA levels, could impact the coordination of these two proteins in transcription repression. For example, it has been found that post-translational modification, especially phosphorylation, plays important roles in regulating the functions of *CDC6* and *BM11* [9–11]. We also cannot rule out the possibility that *BM11* might negatively regulate the *INK4-ARF* locus in unknown *CDC6*-independent ways.

The fundamental findings from our current SCCHN and HARM studies may provide the opportunity to investigate of other cancer types and disease states in order to establish the role of *RD*-mediated regulation of the *INK4-ARF* locus as a universal cellular mechanism [5–7]. However, as described earlier, some of these intriguing observation must be addressed with caution as the cohort size diminishes and specific clinico-pathologic features dichotomize the oral tissue groups: the SCCHN group and the healthy volunteer group were not optimally balanced in regard to age, smoking status, and HPV16 infection, and the small cohort size limited our ability to definitively explore the associations among *RD* status, *BM11* (and/or *CDC6*) expression, statuses and expression of individual *INK4-ARF* genes, and factors like age, smoking status, alcohol use, HPV infection, tumor stage, and tumor location. Ideally, a much larger cohort of patient-matched cancer and premalignant specimens, with a comparable balanced healthy control group would facilitate the comprehensive analyses of genetic alterations in *RD* and in each individual *INK4-ARF* gene. Such studies are essential to fully understand the contributions of *RD*-mediated *en bloc* regulation in oral carcinogenesis, and are currently being pursued by our laboratory. Until such studies are completed, our current work provides a fundamental framework for addressing the interactive roles of the *RD* transcriptional modifier and *INK4-ARF* locus tumor suppressor genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation

ARF	alternative reading frame
BMI1	B lymphoma Mo-MLV insertion region 1 homolog, a polycomb ring finger oncoprotein
CDC6	cell division cycle 6 protein
CDK4/6	cyclin D-dependent kinase 4/6
Cq	quantification cycle value of real-time PCR
FBS	fetal bovine serum
HARM	High At-Risk Mucosa specimens, “phenotypically normal” tissues neighboring but beyond the surgical resection margin of tumor foci
HPRT1	hypoxanthine phosphoribosyltransferase 1
HPV	human papillomavirus
INK4	inhibitors of CDK4
<i>p14</i>, also <i>p14</i>^{ARF}	alternate reading frame of <i>p16</i> ^{INK4a}
<i>p15</i>	<i>p15</i> ^{INK4b}
<i>p16</i>	<i>p16</i> ^{INK4a}
<i>p18</i>	<i>p18</i> ^{INK4c}
PAGE	polyacrylamide electrophoresis
PcG	polycomb group proteins
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
pRb	the retinoblastoma susceptible gene product
RT-qPCR	reverse transcription quantitative real-time PCR
<i>RD</i>, also <i>p14</i>^{ARF}	the regulatory element of the <i>INK4-ARF</i> locus
SCCHN	squamous cell carcinoma of the head and neck

References

1. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. *Cancer Cell*. 2002; 2:103–112. [PubMed: 12204530]
2. Kim WY, Sharpless NE. The regulation of *INK4/ARF* in cancer and aging. *Cell*. 2006; 127:265–75. [PubMed: 17055429]
3. Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim Biophys Acta*. 2002; 1602:73–87. [PubMed: 11960696]
4. Li J, Poi MJ, Tsai M-D. Regulatory mechanisms of tumor suppressor P16^{INK4A} and their relevance to cancer. *Biochemistry*. 2011; 50:5566–5582. [PubMed: 21619050]
5. Gonzalez S, Klatt P, Delgado S, et al. Oncogenic activity of Cdc6 through repression the *INK4/ARF* locus. *Nature*. 2006; 440:702–706. [PubMed: 16572177]

6. Gonzalez S, Serrano M. A new mechanism of inactivation of the *INK4/ARF* locus. *Cell Cycle*. 2006; 5:1382–1384. [PubMed: 16855387]
7. Agherbi H, Gaussmann-Wenger A, Verthuy C, et al. Polycomb mediated epigenetic silencing and replication timing at the *INK4a/ARF* locus during senescence. *PLoS one*. 2009; 4:e5622. [PubMed: 19462008]
8. Borlado LR, Mendez J. CDC6: from DNA replication to cell cycle checkpoints and oncogenesis. *Carcinogenesis*. 2008; 29:237–243. [PubMed: 18048387]
9. Nishitani H, Lygerou Z. Control of DNA replication licensing in a cell cycle. *Genes to Cells*. 2002; 7:523–534. [PubMed: 12059957]
10. Siddique HR, Salem M. Role of BMI1, a stem cell factor, in cancer recurrence and chemoresistance: preclinical and clinical evidences. *Stem Cells*. 2012; 30:372–378. [PubMed: 22252887]
11. Cao L, Bombard J, Cintron K, et al. BMI1 as a novel target for drug discovery in cancer. *J Cell Biochem*. 2011; 112:2729–2741. [PubMed: 21678481]
12. Li, J.; Knobloch, TJ.; Poi, MJ., et al. Genetic alterations of RD^{INK4/ARF} enhancer in human cancer cells. *Mol Carcinog*. 2012. <http://dx.doi.org/10.1002/mc.21965>
13. Li J, Weghorst CM, Tsutsumi M, et al. Frequent *p16^{INK4A}/CDKN2A* alterations in chemically induced Syrian golden hamster pancreatic tumors. *Carcinogenesis*. 2004; 25:263–268. [PubMed: 14604895]
14. Li J, Knobloch TJ, Kresty LA, et al. Gankyrin, an epithelial biomarker, is frequently over-expressed in human oral cancers. *Anticancer Res*. 2011; 31:2683–2692. [PubMed: 21868508]
15. Gillison ML, D'Souza G, Westra W, et al. Distinct risk factors profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. *J of the Natl Cancer Inst*. 2008; 100:407–420.
16. Maitland NJ, Bromidge T, Cox MF, et al. Detection of human papillomavirus genes in human oral tissue biopsies and cultures by polymerase chain reaction. *Br J Cancer*. 1989; 59:698–703. [PubMed: 2544219]
17. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc*. 2008; 3:1101–1108. [PubMed: 18546601]
18. American cancer Society. *Cancer Facts and Figures 2012*. Atlanta, GA: American Cancer Society; 2012.
19. Gandini S, Botteri E, Iodice S, et al. Tobacco smoking and cancer: a meta-analysis. *Intl J of Cancer*. 2008; 122:155–164.
20. Hashibe M, Brennan P, Benhamou S, et al. Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the international Head and Neck Cancer Epidemiology Consortium. *J of the Natl Cancer Inst*. 2007; 99:777–789.
21. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J of Clin Oncol*. 2011; 29:4294–4301. [PubMed: 21969503]
22. Poi MJ, Yen T, Li J, et al. Somatic *INK4a-ARF* locus mutations: a significant mechanism of gene inactivation in squamous cell carcinomas of the head and neck. *Mol Carcinog*. 2001; 30:26–36. [PubMed: 11255261]
23. Jacobs JJ, Kieboom K, Marino S, et al. The oncogene and Polycomb-group *bmi-1* regulates cell proliferation and senescence through the *ink4a* locus. *Nature*. 1999; 397:164–168. [PubMed: 9923679]
24. Krimpendorf P, Ijpenberg A, Song JY, et al. p15Ink4b is a critical tumor suppressor in the absence of p16Ink4a. *Nature*. 2007; 448:943–946. [PubMed: 17713536]
25. Wiedemeyer R, Brennan C, Heffernan TP, et al. Feedback circuit among INK4 tumor suppressors constrains human glioblastoma development. *Cancer Cell*. 2008; 13:355–364. [PubMed: 18394558]
26. Facchino S, Abdouh M, Bernier G. Brain cancer stem cells: current status on glioblastoma multiforme. *Cancers*. 2011; 3:1777–1797. [PubMed: 24212782]

27. Smeds J, Berggren P, Ma X, et al. Genetic status of cell cycle regulators in squamous cell carcinoma of the esophagus: the *CDKN2A* (*p16^{INK4a}* and *p14^{ARF}*) and *p53* genes are major targets for inactivation. *Carcinogenesis*. 2002; 23:645–655. [PubMed: 11960918]
28. Kresty LA, Mallery SR, Knobloch TJ, et al. Frequent alterations of *p16^{INK4a}* and *p14^{ARF}* in oral proliferative verrucous leukoplakia. *Cancer Epidemi Biomark Prev*. 2008; 17:3179–3187.
29. Lang JC, Borchers J, Danahey D, et al. Mutational status of overexpressed *p16* in head and neck cancer: evidence for germline mutation of *p16/p14ARF*. *Int J Oncol*. 2002; 21:401–408. [PubMed: 12118338]
30. Liu Y, Liu F, Yu H, et al. Akt phosphorylates the transcriptional repressor *bmi1* to block its effects on the tumor-suppressor *ink4a-arf* locus. *Sci Signal*. 2012; 5:ra77.10.1126/scisignal.2003199 [PubMed: 23092893]

Figure 1.

Deletions in the *INK4-ARF* locus. **(A)** The schematic structure of the *INK4-ARF* locus. Promoters for *p14*, *p15*, and *p16* genes are indicated by horizontal arrows. Boxes represent exons, and the empty circle represents the regulatory element RD. Sizes of coding regions and non-coding regions are not in proportion strictly. The (-) sign indicates negative regulation; red lines represent the locations of dual-labeled fluorogenic probes used in the deletion analyses. This figure was adapted from Reference 6. **(B)** The calibration curves for qPCR-based deletion assays. qPCR reactions using primers and fluorogenic probes against a target gene and β -actin (*ACTB*, the reference gene) were carried out separately with a series of mixtures of genomic DNAs from TE-1177 (with intact *INK4-ARF* locus) and Panc-1 (with homozygously-deleted *INK4-ARF* locus) cells at various ratios (100:0, 75:25, 50:50, 25:75, 0:100) as previously described [12]. The calibration curves were generated through plotting the Cq values against known ratios of RD (in exponential form). The correlation coefficients of these curves are around -0.99, indicating that the dosage of a target gene in a sample can be accurately measured using this technique [13]. The genetic status of a target gene was determined as follows: dosage <25%, -/-; dosage between 25% and 75%, +/-;

and dosage >75%, +/+. RD, red circle; *p15*, green square; *p14*, yellow triangle; *p16*, blue diamond.

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Figure 2. mRNA expression of *CDC6* and *BMII* in SCCHN, HARM, and healthy control tissues. **A**, *CDC6* expression; **B**, *BMII* expression; **C**, *BMII* expression in tissues with different *RD* statuses. RT-qPCR-based assays using pre-validated primers and probes (Supplementary Table 1) were used to determine the mRNA expression levels of target genes. *HPRT1* was used as an endogenous reference, and the relative expression level of a target gene was defined using the 2^{-Cq} method [16]. Each assay was conducted in triplicate and the average value was used for statistical analyses. Data were presented in box plots. Differences in *CDC6* or *BMII* mRNA expression between the control group and the SCCHN or HARM group were analyzed using two-sided Welch two-sample *t*-tests with the significant level of

$p = 0.05$, whereas the difference between the SCCHN group and the HARM group was analyzed using two-sided paired t -tests. RD^- HARM, HARM specimens with homozygous and heterozygous RD deletions; RD^- Tumor, SCCHN specimens with homozygous and heterozygous RD deletions; RD^+ HARM, HARM specimens with intact RD ; RD^+ Tumor, SCCHN specimens with intact RD . *, $p < 0.05$; **, $p < 0.01$.

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Figure 3. mRNA expression of *p16* (A), *p14* (B), and *p18* (C) in SCCHN, HARM, and healthy control tissues. Legends are similar as in Fig. 2. For *p16* and *p14*, differences between different groups were analyzed using two-sided Welch two-sample *t*-tests with the significant level of $p = 0.05$. For *p18*, differences between the control group and the SCCHN or HARM group were analyzed as *p16* or *p14*, whereas the difference between the SCCHN group and the HARM group was analyzed using two-sided paired *t*-tests. *, $p < 0.05$; **, $p < 0.01$.

Table 1

Demographics and clinicopathologic features of patients and healthy controls

	No. of Patients (%)	No. of Healthy Controls (%)	<i>p</i> -value*
Gender			1.0
Male	22 (73.3%)	19 (73.1%)	
Female	8 (26.7%)	7 (26.9%)	
Race			0.12
White	29 (96.7%)	21 (80.8%)	
Black	1 (3.3%)	2 (7.7%)	
Asia	0	3 (11.5%)	
Ethnicity			0.21
Hispanic/Latin	0 (0%)	2 (7.7%)	
Non Hispanic/Latin	30 (100%)	24 (92.3%)	
Age			0.001
Average	56.6	40.1	
Range	37–79	22–79	
< 45	6 (20%)	16 (61.5%)	
45–60	13 (43.3%)	9 (34.6%)	
>60	11 (36.7%)	1 (3.8%)	
Tumor Location			
Floor of mouth	5 (16.7%)		
Tongue	20 (66.7%)		
Others	5 (16.6%)		
Tumor Stage**			
I	8 (26.7%)		
II	8 (26.7%)		
III	5 (16.7%)		
IV	8 (26.7%)		
HPV16 Infection			0.055
–	25 (83.3%)	26 (100%)	
+	5 (16.7%)	0 (0%)	
Smoking Status			0.015
Smoker**	21 (70%)	9 (34.6%)	
Non-smoker	9 (30%)	17 (65.4%)	
Alcohol Use Status			0.56
User***	23 (76.7%)	18 (69.3%)	
Non-user	7 (23.3%)	8 (30.7%)	

* Fisher exact tests or Pearson's χ^2 tests.

** The tumor stage information for one patient was missing.

Including both current and former tobacco/alcohol users.

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Table 2

Deletions of the *INK4-ARF* locus in SCCN, HARM, and healthy control specimens*

	RD	p15	p14	p16	HPV16		RD	p15	p14	p16	HPV16		RD	p15	p14	p16	HPV16
04T1	+/+	+/+	+/+	+/+	-	04H1	+/+	+/+	+/+	+/+	-	C1	+/+	+/+	+/+	+/+	-
06T1	+/+	+/+	+/+	+/+	-	06H1	+/+	+/+	+/+	+/+	-	C2	+/+	+/+	+/+	+/+	-
07T1	+/+	+/+	+/+	+/+	-	07H1	+/+	+/+	+/+	+/+	-	C3	+/+	+/+	+/+	+/+	-
08T1	+/+	+/+	+/+	+/+	-	08H1	+/+	+/+	+/+	+/+	-	C4	+/+	+/+	+/+	+/+	-
09T1	+/+	+/+	+/+	+/+	-	09H1	+/+	+/+	+/+	+/+	-	C5	+/+	+/+	+/+	+/+	-
10T1	+/+	+/+	+/+	+/+	+	10H1	+/+	+/+	+/+	+/+	-	C6	+/+	+/+	+/+	+/+	-
11T1	+/+	+/+	+/+	+/+	-	11H1	+/+	+/+	+/+	+/+	-	C7	+/+	+/+	+/+	+/+	-
13T1	+/+	+/+	+/+	+/+	-	13H1	+/+	+/+	+/+	+/+	-	C8	+/+	+/+	+/+	+/+	-
14T1	+/+	+/+	+/+	+/+	-	14H1	+/+	+/+	+/+	+/+	-	C9	+/+	+/+	+/+	+/+	-
16T1	+/+	+/+	+/+	+/+	-	16H1	+/+	+/+	+/+	+/+	-	C10	+/+	+/+	+/+	+/+	-
17T1	+/+	+/+	+/+	+/+	-	17H1	+/+	+/+	+/+	+/+	-	C11	+/+	+/+	+/+	+/+	-
18T1	+/+	+/+	+/+	+/+	-	18H1	+/+	+/+	+/+	+/+	-	C12	+/+	+/+	+/+	+/+	-
19T1	+/+	+/+	+/+	+/+	-	19H1	+/+	+/+	+/+	+/+	-	C13	+/+	+/+	+/+	+/+	-
20T1	+/+	+/+	+/+	+/+	-	20H1	+/+	+/+	+/+	+/+	+	C14	+/+	+/+	+/+	+/+	-
21T1	+/+	+/+	+/+	+/+	+	21H1	+/+	+/+	+/+	+/+	-	C15	+/+	+/+	+/+	+/+	-
22T1	+/+	+/+	+/+	+/+	-	22H1	+/+	+/+	+/+	+/+	-	C16	+/+	+/+	+/+	+/+	-
23T1	+/+	+/+	+/+	+/+	+	23H1	+/+	+/+	+/+	+/+	-	C17	+/+	+/+	+/+	+/+	-
24T1	+/+	+/+	+/+	+/+	-	24H1	+/+	+/+	+/+	+/+	-	C18	+/+	+/+	+/+	+/+	-
25T1	+/+	+/+	+/+	+/+	-	25H1	+/+	+/+	+/+	+/+	-	C19	+/+	+/+	+/+	+/+	-
26T1	+/+	+/+	+/+	+/+	-	26H1	+/+	+/+	+/+	+/+	-	C20	+/+	+/+	+/+	+/+	-
28T1	+/+	+/+	+/+	+/+	-	28H1	+/+	+/+	+/+	+/+	-	C21	+/+	+/+	+/+	+/+	-
29T1	+/+	+/+	+/+	+/+	-	29H1	+/+	+/+	+/+	+/+	+	C22	+/+	+/+	+/+	+/+	-
30T1	+/+	+/+	+/+	+/+	-	30H1	+/+	+/+	+/+	+/+	+	C23	+/+	+/+	+/+	+/+	-
31T1	+/+	+/+	+/+	+/+	-	31H1	+/+	+/+	+/+	+/+	-	C24	+/+	+/+	+/+	+/+	-
32T1	+/+	+/+	+/+	+/+	+	32H1	+/+	+/+	+/+	+/+	+	C25	+/+	+/+	+/+	+/+	-
33T1	+/+	+/+	+/+	+/+	+	33H1	+/+	+/+	+/+	+/+	-	C26	+/+	+/+	+/+	+/+	-

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	RD	p15	p14	p16	HPV16		RD	p15	p14	p16	HPV16		RD	p15	p14	p16	HPV16
	+/+	+/+	+/+	+/+	-		+/+	+/+	+/+	-/-	-						
34T1	+/+	+/+	+/+	+/+	-	34H1	+/+	+/+	+/+	+/+	-						
36T1	+/+	+/+	+/+	+/+	-	36H1	+/+	+/+	+/+	+/+	-						
37T1	+/+	+/+	+/+	+/+	-	37H1	+/+	+/+	+/+	+/+	-						
38T1	+/+	+/+	+/+	+/+	-	38H1	+/+	+/+	+/+	+/+	-						

* T, SCCHN tumor; H, HARM; C, healthy control. +/+, wild type; +/-, heterozygous deletion; -/-, homozygous deletion.

Table 3
Deletion events in the *INK4-ARF* locus in SCCHN, HARM, and healthy control specimens

	RD deletion	<i>p15</i> deletion	<i>p14</i> deletion	<i>p16</i> deletion	Deletion in the <i>INK4-ARF</i> locus *
SCCHN	11 (11/30)	13 (13/30)	7 (7/30)	7 (7/30)	17 (17/30)
HARM	4 (4/30)	1 (1/30)	3 (3/30)	3 (3/30)	7 (7/30)
Normal	0 (0/26)	0 (0/26)	0 (0/26)	0 (0/26)	0 (0/26)
	Frequency Comparison **				
SCCHN vs HARM	<i>p</i> = 0.07	<i>p</i> < 0.01	<i>p</i> = 0.30	<i>p</i> = 0.30	<i>p</i> = 0.017
SCCHN vs Normal	<i>p</i> < 0.01	<i>p</i> < 0.01	<i>p</i> = 0.012	<i>p</i> = 0.012	<i>p</i> < 0.01
HARM vs Normal	<i>p</i> = 0.12	<i>p</i> = 1	<i>p</i> = 0.24	<i>p</i> = 0.24	<i>p</i> = 0.012

* Specimens containing deletion(s) in at least one of RD, *p15*, *p14*, and *p16*.

** Fisher's exact tests.