Coordinated Expression of Cyclin-dependent Kinase-4 and its Regulators in Human Oral Tumors

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Abstract. *Background/Aim: While aberrant expression of cyclin-dependent kinase-4 (CDK4) has been found in squamous cell carcinoma of the head and neck (SCCHN), the associations between CDK4 and its regulators, namely, cyclin D1, cyclin E, gankyrin, SEI1, and BMI1 in gene expression remain to be explored. Herein we investigated the mRNA profiles of these oncogenes and their interrelations in different oral lesion tissues. Materials and Methods: Thirty SCCHN specimens and patient-matched high at-risk mucosa (HARM) and 16 healthy control specimens were subjected to quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses. Results: The mRNA levels of CDK4, cyclin D1, gankyrin, SEI1, BMI1 were significantly elevated in both HARM and SCCHN (in comparison with control specimens), and statistically significant correlations were found among these markers in gene expression. Conclusion: Up-regulation of CDK4 and its regulators takes place in oral cancer progression in a coordinate manner, and HARM and SCCHN share a similar molecular signature within the CDK4-pRB pathway.*

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Carcinogenesis is a multi-step process through which normal cells are sequentially transformed *via* the activation of proto-oncogenes and inactivation of tumor suppressor genes into their malignant derivatives, and most of these tumor-prone events occur in cellular signaling pathways involving p53 (the *TP53* gene product) and pRb (the *RB1* gene product) (1-4). While p53 acts as a pivotal regulator in apoptosis and senescence (2, 5), pRb functions primarily in cell proliferation, especially in progression through the G_1-to-S transition of the cell cycle (1, 4). In G_0 and early G_1 phases, pRb is hypo-phosphorylated and forms complexes with members of the E2F family of transcription factors. These complexes sequester E2Fs and prevent their access to the promoters of proliferation-associated genes, such as cyclin B1 (*CCNB1*) and jun B proto-oncogene (*JUNB*) (1). Once committed to cell proliferation in late G_1, pRb is progressively hyper-phosphorylated by cyclin-dependent kinases-4 and -6 (CDK4/6; CDK4 is the primary kinase), resulting in the entry into S phase (6). Consequently, CDK4-mediated phosphorylation of pRb is under strict homeostatic controls in order to maintain biomolecular equilibrium and promote cell cycle progression.

Intricate molecular machinery and mechanisms are present in cells to modulate pRb phosphorylation. On one hand, $p16^{INK4A}$ (p16), a member of the INK4 inhibitors (specific inhibitors of CDK4, including $p15^{INK4B}$, p16, $p18^{INK4C}$, and $p19^{INK4D}$), binds to CDK4 and inhibits its kinase activity, thus maintaining pRb in its hypo-phosphorylated E2F-associated state (6). On the other hand, CDK4 itself exhibits minimal pRb-phosphorylating activity and becomes fully functional only after being charged with cyclins, especially cyclin D1, D2,

and D3 (1). Moreover, it has been reported that a number of proteins are able to up-regulate CDK4 in cells through different mechanisms (1). For example, gankyrin competes with p16 for binding to CDK4, thus blocking the CDK4-inhibitory activity of p16 (7); $p34^{SEI1}$ (*SERTAD1*) is able to bind to the CDK4-cyclin D1 complex and enhance the latter's kinase activity (8, 9); BMI1 (B lymphoma Mo-MLV insertion region 1 homolog, a polycomb ring finger protein) down-regulates the expression of *p16* (*CDKN2A*) and *p15* (*CDKN2B*) genes, which consequently promotes CDK4-mediated phosphorylation of pRb (10). In addition, while cyclin D1 physically interacts with both CDK4 and CDK2 in cells, it has been reported that in some tumor cells, elevated expression of cyclin E, a cyclin primarily binding to CDK2, is able to influence the distribution of cyclin D1 between CDK4 and CDK2 in favor of CDK4-mediated phosphorylation of pRb (11, 12).

In normal cells, all the aforementioned positive and negative regulators work coordinately to ensure the control at the G_1-S transition (1). Nonetheless, in most human malignancies, the balance among these regulators is impaired through diverse mechanisms, ultimately leading to the de-regulation of the pRb pathway and aberrant cell proliferation (2). While genetic inactivation of the *CDKN2A/p16* gene has been found in nearly 50% of all human cancers (4), over-expression of cyclin D1 (13, 14), gankyrin (15, 16), $p34^{SEI1}$ (17-19), and BMI1 (20, 21) has been found prevalently in breast, lung, liver cancers and oral squamous cell carcinoma. In regard to the fact that all these molecular events have the potential to impact CDK4-mediated phosphorylation of pRb in cancer cells, the interesting question is whether/how these molecular events are coordinated with each other in carcinogenesis. In a recent study (22) on a cohort of 30 squamous cell carcinomas of the head and neck (SCCHN) and patient-matched high at-risk mucosa (HARM, phenotypically "normal" tissues beyond the surgical resection margin and distant to the SCCHN foci), we found a positive association between down-regulation of *p16* mRNA and up-regulation of *BMI1* in SCCHN specimens, supporting the model that alterations in CDK4-related tumor suppressors (p16) and oncoproteins (BMI1) are coordinated in SCCHN. In the present study, we continued to investigate the potential correlations among alterations in the mRNA levels of *CDK4, CCND1/cyclin D1* (hereafter, cyclin D1) *CCNE1/cyclin E* (*cyclin E*) *PSMD10/gankyrin* (*gankyrin*), *SERTAD1/p34SEI1* (*SEI1*), and *BMI1* oncogenes in SCCHN, HARM, and healthy controls. Our results revealed coordinated over-expression of these oncogenes in SCCHN and HARM. Further studies using principal component analyses (PCA) showed that the mRNA expression profiles of *CDK4, cyclin D, cyclin E, gankyrin, SEI1*, and *BMI1* in SCCHN and HARM are distinct from those in healthy controls, but not between themselves, indicating that in spite of their differences in histology, HARM and SCCHN are similar in some molecular biological characteristics.

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 (16, 23). Additional oral brush biopsy specimens from 16 healthy donors were collected using an OralCDx brush (Oral Cancer Prevention International; Suffern, NY) and used for DNA and RNA isolation.

Detection of HPV16 E6 in oral tissue biopsies. Since HPV16 is the major HPV subtype in US that infects oral tissues, a PCR-based assay was performed, as previously described to detect the presence of *HPV16 E6* gene in oral specimens (24). Briefly, PCR reactions were carried-out for 30 cycles of denaturation (30 sec at 94˚C), annealing (30 sec at 60˚C) and extension (30 sec at 72˚C) using primers specific for *HPV16 E6*: forward, 5'TCAAAAGCCACTG TGTCCTG3'; reverse, 5'CGTGTTCTTGATCTGCA3'. The PCR product (120 bp) was subjected to 10% PAGE electrophoresis and visualized using ethidium bromide.

Quantitative determination of the mRNA expression levels of CDK4, cyclin D1, cyclin E, gankyrin, SEI1, and BMI1. RT-qPCR reactions were carried-out using pre-validated TaqMan gene expression assays as following: Hs00175935_m1 for *CDK4*, Hs00277039_m1 for *cyclin D1*, Hs0023356_m1 for *cyclin E*, Hs00829508-s1 for *gankyrin*, Hs00203547_m1 for *SEI1*, Hs00409825_g1 for *BMI1*, and Hs99999909_m1 for human hypoxanthine phosphoribosyltransferase (*HPRT1*). Of note, *HPRT1* was used as an endogenous reference for normalized gene expression. 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 (25).

Statistical analyses. All data in the present 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 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 to compare 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. Principal component analyses (PCA) were applied to evaluate the differences among SCCHN, HARM, and healthy controls in the mRNA levels of selected oncogenes. All tests are two-sided and the significance level was at α=0.05.

Results

Patients' clinico-pathological features. Thirty SCCHN patients and 16 healthy volunteers were included in the present study. All participants were eligible and were acquired as part of an enrollment process without any restriction on gender, race, age, and ethnicity. Demographics and clinico-pathological features of these

cancer patients and healthy controls are summarized in Table I. There are no significant differences between these two groups with respect to sex, race, and ethnicity. Notably, there are more men than women in the SCCHN patient group as well as in the healthy control group, which is consistent with the observation that men are more likely to be diagnosed with SCCHN (26). In addition, there are more white participants than people from other races in both groups. However, there exists a significant difference between these two groups in age ($p<0.01$) at the time of tissue collection. Specifically, while only one healthy participant was over 60 (1/16, 6.25%), 11/30 SCCHN patients (36.7%) were in the same age group. Contrarily, both groups had six healthy participants under the age of 45 (37.5% and 20%, respectively). It is well-documented that most of SCCHN patients are between 50-70 years old (26), hence, the age distribution of SCCHN patients involved in our current study is likely a reflection of typical SCCHN patient characteristics.

Tobacco use (typically cigarette use), alcohol consumption, and human papillomavirus (HPV) infection are three primary risk factors of SCCHN (22, 26). As shown in Table I, the SCCHN group and the healthy-volunteer group appear to be different from each other ($p=0.058$) regarding smoking status. While the majority of SCCHN patients were ever-smokers (current or former) (70%, 21/30), only 6 out of 16 healthy controls (37.5%) consumed cigarettes. As for alcohol consumption, the incidences of current or former drinkers in the SCCHN and healthy control groups are comparable (76.7% *vs*. 62.5%; $p=0.33$). Furthermore, 16.6% of SCCHN specimens (5/30) demonstrated evidence of HPV16 infection, whereas no evidence of HPV16 infection was detected in any of healthy control tissues (0/16). However, the difference between these two groups in the incidence of HPV16 infection is not statistically significant ($p=0.15$).

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.

mRNA expression of CDK4, cyclin D1, cyclin E, gankyrin, SEI1, and BMI1 in oral specimens. We first evaluated the mRNA expression levels of *CDK4, cyclin D1, cyclin E, gankyrin, SEI1,* and *BMI1* in healthy oral tissues, SCCHN and patient-matched HARM specimens using pre-validated Taqman gene expression assays. As shown in Figure 1A, *CDK4* was significantly up-regulated in HARM ($p<0.01$) and SCCHN (to a lesser extent with $p=0.06$) (in comparison with the healthy volunteer group). Moreover, there is virtually no difference in *CDK4* mRNA expression between the SCCHN and HARM groups, indicating that up-regulation of *CDK4* is a molecular event occurring in the very early stage of oral carcinogenesis. Similar patterns are observed in the expression profiles of *cyclin D1* (Figure 1B), *gankyrin* (Figure 2A), *SEI1* (Figure 2B), and *BMI1* (Figure 2C): each of these genes was expressed at significantly elevated mRNA levels in SCCHN and HARM, however, their expression level in HARM was comparable to that in SCCHN. In contrast, while no substantial difference in the mRNA level of *cyclin E* is observed between the healthy control group and HARM (Figure 1C), the mRNA level of *cyclin E* in SCCHN is considerably higher than that in the healthy control group ($p=0.02$), implying that up-regulation of *cyclin E* occurs in the late stage (*i.e*. SCCHN) of oral cancer development.

Table I. *Demographics and clinico-pathological features of patients and healthy controls*.

	No. of patients (%)	No. of healthy controls (%)	*p*-Value*
Gender			0.74
Male	22 (73.3%)	11 (68.8%)	
Female	8 (26.7%)	5 (31.2%)	
Race			0.43
White	29 (96.7%)	14 (87.5%)	
Black	1 (3.3%)	1 (6.25%)	
Asia	0	1 (6.25%)	
Ethnicity			0.35
Hispanic/Latin	0 (0%)	1 (6.25%)	
Not Hispanic/Latin	30 (100%)	15 (93.75%)	
Age			0.0088
Average	56.6	47.1	
Range	37-79	29-79	
<45	6 (20%)	6 (37.5%)	
45-60	13 (43.3%)	9 (56.25%)	
>60	11 (36.7%)	1 (6.25%)	
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			0.15
–	25 (83.3%)	16 (100%)	
+	5 (16.7%)	0 (0%)	
Smoking Status			0.058
Smoker**	21 (70%)	6 (37.5%)	
Non-smoker	9 (30%)	10 (62.5%)	
Alcohol Use Status			0.33
User***	23 (76.7%)	10 (62.5%)	
Non-user	7 (23.3%)	6 (37.5%)	

*Fisher's 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.

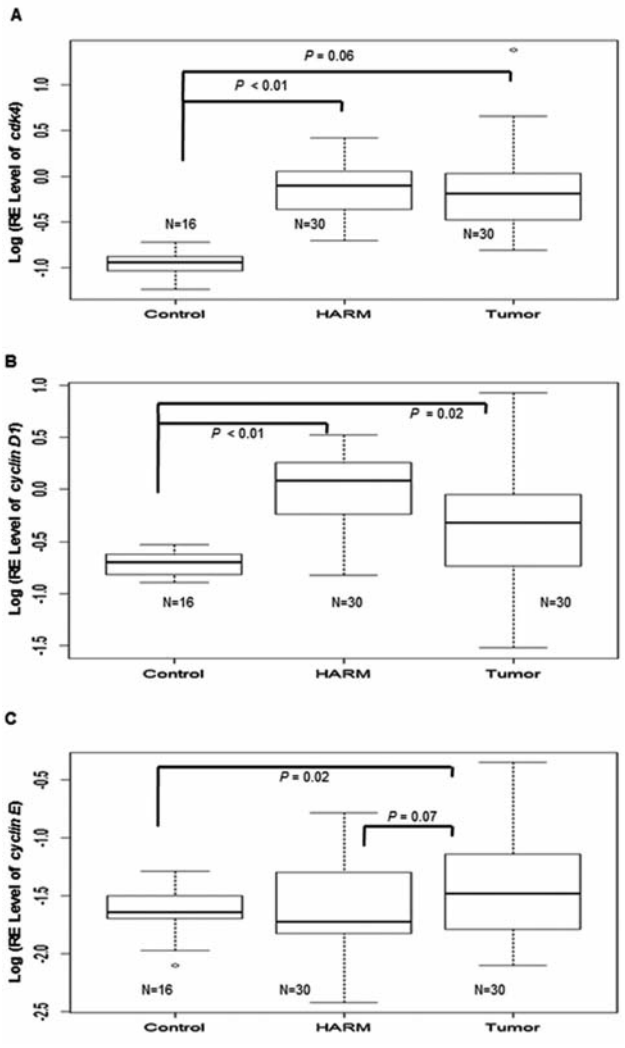

Figure 1. *mRNA Expression of CDK4, cyclin D1, and cyclin E in SCCHN, HARM, and healthy control tissues. A, CDK4 expression; B, cyclin D1 expression; C, cyclin E expression. Pre-validated qRT-PCR-based assays were used to evaluate the mRNA expression levels of target genes. HPRT1 was used as an endogenous reference, and the relative expression (RE) level of a target gene was defined using the 2−ΔCq method (25). Each assay was conducted in triplicate and the average value was used for statistical analyses. Data were presented in box plots. Differences in 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.*

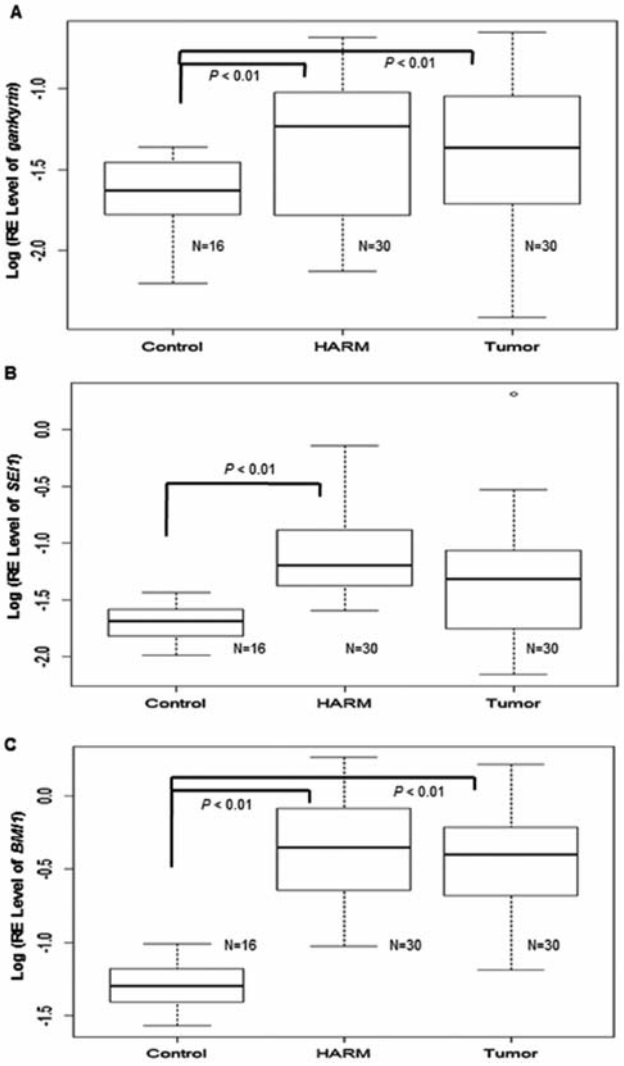

Figure 2. *mRNA Expression of gankyrin (A), SEI1 (B), and BMI1 (C) in SCCHN, HARM, and healthy control tissues. Legends are similar as in Figure 1.*

We also analyzed the potential correlations between the mRNA expression of the aforementioned oncogenes and those demographic and clinico-pathological factors, such as age, tumor location, tumor stage, smoking, alcohol consumption, and HPV16 infection. Our results do not support any significant association between the mRNA expression of any of these oncogenes and the aforementioned demographic and clinico-pathological factors (data not shown).

Coordination among CDK4, cyclin D1, cyclin E, gankyrin, SEI1, and BMI1 in mRNA expression. We then analyzed the interrelations between the aforementioned six oncogenes in mRNA expression. Table II summarizes the Pearson's correlation coefficients between any two of these markers in different oral specimens. In healthy control tissues, statistically significant correlations were found between *CDK4:gankyrin, CDK4:SEI1, cyclin D1:gankyrin, cyclin D1:SEI1,* and *gankyrin:SEI1*. For *CDK4, cyclin D1, gankyrin,* and *SEI1,* their overall mRNA levels were relatively low in

Table II. *Correlation coefficients between CDK4, cyclin D1, cyclin E, gankyrin, SEI1, BMI1, and CDC6 in mRNA expression in different oral specimens*[†].

	CDK4	*cyclin D1*	*cyclin E*	*Gankyrin*	*SEI1*	*BMI1*
SCCHN						
CDK4	1.0000					
cyclin D1	0.8447**	1.000				
cyclin E	0.7821**	0.7144**	1.000			
gankyrin	0.5096**	0.4257*	0.5095**	1.000		
SEI1	0.9879**	0.8412**	0.8217**	0.5198**	1.000	
BMI1	0.1904#	0.2235	0.2656	0.4220*	0.1423	1.000
HARM						
CDK4	1.000					
cyclin D1	0.8026**	1.000				
cyclin E	0.4985**	0.5068**	1.000			
gankyrin	0.2407	0.3729*	0.1518	1.000		
SEI1	0.5228**	0.4930**	0.9186**	0.0692	1.000	
BMI1	0.3850*	0.2052	–0.2227	0.4988**	–0.1714	1.000
Control						
CDK4	1.000					
cyclin D1	0.4594	1.000				
cyclin E	0.1505	0.0969	1.000			
gankyrin	0.6715**	0.6323**	0.2846	1.000		
SEI1	0.9669**	0.6287**	0.0557	0.7195**	1.000	
BMI1	–0.1054	0.3916	0.1855	–0.0851	–0.0637	1.000

[†]Pearson's correlation coefficients (r) were listed here. All coefficients were analyzed using Pearson's product-moment correlation tests, and the *p* values from these tests were marked in different colors: #$p<0.10$; *$p<0.05$; **$p<0.01$.

healthy control tissues (in comparison to SCCHN and HARM; Figures 2 and 3), which may contribute to the observed "positive" associations between these markers.

In HARM, positive correlations emerged between *CDK4:cyclin D1, CDK4:cyclin E, cyclin D1:cyclin E, cyclin E:SEI1, CDK4:BMI1, gankyrin:BMI1*. Interestingly, the associations of *CDK4:gankyrin* and *SEI1:gankyrin* observed in healthy control tissues were not found in HARM.

In SCCHN, these six markers were widely associated in mRNA expression as observed in HARM but to a wider extent. Besides those correlations observed in HARM, *gankyrin* was positively associated with each of *CDK4*, *cyclin E,* and *SEI1* in mRNA expression in SCCHN, while the corresponding correlations were not observed in HARM.

Discussion

Disruption of the CDK4-pRb pathway occurs frequently in a variety of human cancers, and in many cases, the underlying molecular mechanisms involve the activation of *CDK4* gene as well as CDK4-promoting oncogenes or the inactivation of CDK4-inhibiting tumor suppressor genes or both, resulting in aberrant CDK4-mediated phosphorylation of pRb (1, 4). Herein we investigated the mRNA expression profiles of five distinct CDK4-promoting factors in different oral specimens: *cyclin D1, cyclin E, gankyrin, SEI1,* and *BMI1*. Even though the modulation of these factors on CDK4 is mainly through protein-protein interactions, our studies on the interrelations between these factors (as well as CDK4) in mRNA expression in SCCHN and patient-matched HARM specimens provide novel insights into understanding the roles of CDK4 and its regulators in oral carcinogenesis.

Up-regulation of CDK4 and its regulators. Previous studies in our laboratory have shown that mRNA levels of *gankyrin*, *SEI1*, and *BMI1* are elevated in human SCCHN specimens at considerable frequencies (16, 22, 28). These findings are further supported by results from our current study. Instead of evaluating the mRNA expression profile of a single oncogene, we concurrently analyzed the mRNA levels of six markers (CDK4 and five regulators) in the same cohort of oral specimens in order to generate an expression signature of molecular risk. According to their expression signature in healthy, HARM, and SCCHN individuals, these six markers segregate into two groups. The first group consists of *CDK4, cyclin D1, gankyrin, SEI1*, and *BMI1*. The mRNA levels of these markers are substantially up-regulated during the early initiating stage of oral cancer development, *i.e*. HARM, and remain statistically unchanged in SCCHN. Hence, over-expression of each of *CDK4, cyclin D1, gankyrin, SEI1,* and *BMI1* may be an early indicator of oral carcinogenesis. The second group includes *cyclin E*. The mRNA level of *cyclin*

E significantly elevates only in SCCHN (in comparison with healthy tissues and HARM), suggesting that up-regulation of *cyclin E* could be a late stage marker in oral carcinogenesis.

The mechanisms underlying up-regulation of these markers in SCCHN and HARM remain to be further elucidated. In some cases, gene amplification contributes to or even could be the primary mechanism for activation of specific oncogenes (29); in most cases, the transcription of these oncogenes is enhanced through various known/unknown mechanisms (1). For example, the frequency of amplification of the *cyclin D1* gene in human breast cancer is about 15-20%; however, over-expression of cyclin D1 protein has been found in 50-70% of breast tumor specimens (30). In addition, while the *SEI1* gene is located in human chromosome 19q13.1, a region frequently amplified in human breast, esophagus, ovarian, lung, and pancreatic cancers (8, 17), it has been reported that environmental factors such as UV light or nutrients can induce the expression of *SEI1* as well (8, 31).

Coordination among these markers in oral carcinogenesis. As addressed earlier, aberrant CDK4-mediated phosphorylation of pRb in cancer cells could arise from loss of the coordination among those positive and negative CDK4 regulators. For example, over-expression of p16 has the potential to counteract against up-regulation of CDK4, cyclin D1, and gankyrin. Presumably, the CDK4-pRb pathway may remain functionally intact in a specimen containing elevated CDK4 and p16. However, in the presence of over-expression of CDK4 (or cyclin D1), genetic inactivation of p16 or transcriptionally silencing p16 through up-regulation of BMI1 (and/or CDC6) impairs the CDK4-pRb pathway in a synergic manner, which consequently leads to carcinogenesis (1, 22). From this perspective, it is anticipated that changes in CDK4 and its regulators occur coordinately in the development of human cancers. Such premise has been supported by results from our current study. As shown in Table II, statistically significant correlations are present between *CDK4* and four regulators (*cyclin D1, cyclin E, gankyrin,* and *SEI1*) in mRNA expression in SCCHN ($p<0.05$). The mRNA level of *BMI1* also tends to be associated with that of *CDK4* in SCCHN ($p<0.1$). Intriguingly, *CDK4* and its regulators (except *cyclin E1*) all are up-regulated at the mRNA level in HARM, and the correlations observed in SCCHN (except the one between *CDK4* and *gankyrin*) are also present in HARM, suggesting that changes in *CDK4* and its regulators at the mRNA level occur almost simultaneously.

There are also dependent statistical associations among the selected CDK4 regulators in mRNA expression in SCCHN, including the correlations of cyclin *D1:gankyrin, cyclin D1:SEI1,* and *cyclin E:SEI1*. While some of these observed correlations may only arise from their correlations with CDK4

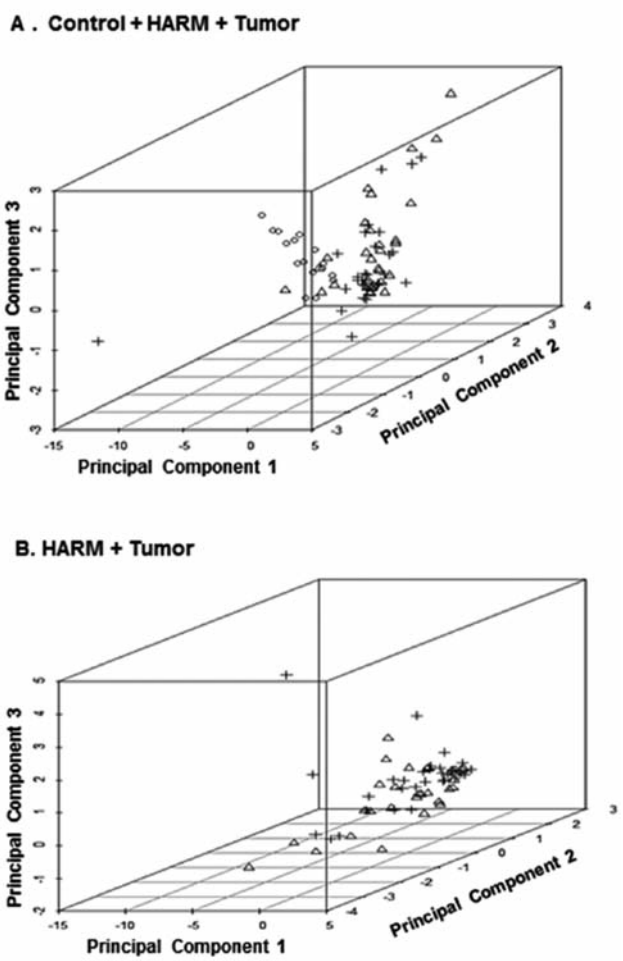

Figure 3. *Principal component analyses (PCA) of the mRNA expression of CDK4, cyclin D1, cyclin E, gankyrin, SEI1, and BMI1 in oral specimens. (A) SCCHN, HARM, and healthy control specimens; (B) SCCHN and HARM specimens. In both A and B, circles (o), triangles (Δ), and plus signs (+) represent health controls, HARM, and SCCHN, respectively. In A, principal components 1, 2, 3 cover 50.0%, 19.4%, and 13.3% of variance. In B, principal components 1, 2, 3 cover 58.9%, 18.6%, and 9.8% of variance.*

or from their correlations with the cell proliferation status in SCCHN, some may be physiologically significant. For example, it has been reported that $p34^{SEI1}$ exhibits a transactivating activity to enhance the transcription of *cyclin E* in cells (31), which could underlie the correlation between these two factors in mRNA expression in HARM and SCCHN. Nonetheless, the physiological mechanisms of these correlations in oral cancers need to be further explored.

Similarities between HARM and SCCHN in molecular biology. An important feature in our current study is that patient-matched HARM specimens were investigated along with SCCHN and healthy control tissues. HARM tissues

represent grossly non-pathogenic "normal" tissues beyond and distant from the surgical resection margin of the SCCHN malignant lesion (22). Due to the prevalence of field cancerization associated with oral epithelial carcinogenesis (32-35), molecular field defects are often acquired in clinically normal-appearing tissues. It is important in this context that our results demonstrate that indeed distant HARM specimens are similar to SCCHN specimens in many molecular biology characteristics without presenting as manifest premalignant or malignant lesions. While *CDK4, cyclin D1, gankyrin, SEI1*, and *BMI1* oncogenes are highly expressed in both HARM and SCCHN (in comparison with true healthy control tissues), the interrelations between these oncogenes in mRNA expression in HARM are similar to those in SCCHN. Moreover, as shown in Figure 3, PCA demonstrate that HARM and SCCHN are distinct from the healthy control group in mRNA profiles of *CDK4* and its selected five regulators but undistinguishable from each other using these discriminators. These findings are consistent with a pattern of oral epithelial carcinogenesis whereby there are many shared molecular defects during premalignant progression, and that once cells are sufficiently disrupted from homeostatic control mechanisms, a few number of changes promote the cells from pr-emalignant to malignant state (33, 35). In addition, our previous studies have shown that deletion of the tumor-suppressive *INK4-ARF* locus occurs in HARM at a considerably high frequency even though such frequency is lower than that in SCCHN (22). Taken together, with regard to the presence of the aforementioned cancer-prone molecular events, HARM, as its name implies, remains highly at-risk and susceptible to molecular carcinogenesis independent of SCCHN tumors, which may reasonably contribute to the high frequency of the recurrence of SCCHN (about 40%) (36, 37). Therefore, proper therapeutic or preventative treatment on HARM may be of significance in clinic.

In conclusion, we have demonstrated that *CDK4, cyclin D1, cyclin E, gankyrin, SEI1*, and *BMI1* oncogenes are up-regulated in a coordinated manner in the development of oral cancers and these molecular events occur even at the very early, "initial" stage, *i.e*. HARM. However, as described earlier, some of those intriguing observations must be addressed with caution due to the relatively small cohort size, the imbalance between the healthy control group and SCCHN group (in regard to age and smoking status as well as HPV infection), unavailability of protein expression data. The small cohort size also limited our ability to explore the association between the expression of CDK4 and its regulator and clinical characters (such as tumor stage, tumor location). More extensive studies using much larger cohorts of patient-matched oral cancer and pre-malignant specimens are essential to fully-understand the interrelations among CDK4 and its regulators in the development of oral cancers.

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