Gankyrin, A Biomarker for Epithelial Carcinogenesis, Is Overexpressed in Human Oral Cancer

JUNAN LI[1,2,*], THOMAS J. KNOBLOCH[1,2,*], LAURA A. KRESTY[3,4], ZHAOXIA ZHANG[1], JAS C. LANG[2,5], DAVID E. SCHULLER[5] and CHRISTOPHER M. WEGHORST[1,2]

[1]*Division of Environmental Health Sciences, College of Public Health,*
[2]*Comprehensive Cancer Center and Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, and* [5]*Department of Otolaryngology, The Ohio State University, Columbus, OH43210, U.S.A.;*
[3]*Sylvester Comprehensive Cancer Center, and*
[4]*Department of Epidemiology and Public Health, Miller School of Medicine, The University of Miami, Miami, FL33101, U.S.A.*

Abstract. *Little is known about the potential involvement of the oncoprotein gankyrin in human oral cancer progression. In this study, the levels of gankyrin mRNA and protein expression were assessed in human oral epithelial cell lines, at-risk normal oral tissues, premalignant oral lesions, and primary oral squamous cell carcinomas (OSCCs). Materials and Methods: Biopsies included 6 oral epithelial cell lines, 32 OSCC specimens for qRT-PCR analysis, 27 OSCC specimens and 12 premalignant oral lesions for immunohistochemical analysis. Results: Gankyrin was overexpressed in all tested oral epithelial cell lines and the majority of OSCC specimens (32/32 (100%) and 21/27 (71%) at the mRNA and protein levels, respectively). Moreover, 6/12 of premalignant oral lesions overexpressed gankyrin protein. Conclusion: Gankyrin overexpression is a prevalent event in human oral cancer and occurs during the early stages of oral carcinogenesis, thus being a viable therapeutic or chemopreventive target in oral cancer.*

Carcinogenesis is a multistep process through which normal cells are sequentially transformed *via* the activation of proto-oncogenes and inactivation of tumor suppressor genes into their malignant counterparts. During human oral cancer development, one of the most prominent genetic changes is alteration of the $p16^{CDKN2A}$ tumor suppressor gene. More than 80% of human oral squamous cell carcinomas (OSCCs) (1, 2) and 50% of severe oral dysplasias (3) have been shown to exhibit *CDKN2A* alterations. Inactivation of the p53 tumor suppressor gene (*TP53*) is another frequent alteration observed in OSCC development and affects nearly 50% of all cases (4). In contrast to the strikingly prevalent incidence of alterations in these important tumor suppressor genes, knowledge related to the activation of specific proto-oncogenes in human oral cancer remains relatively limited.

Recently, overexpression of a newly defined oncoprotein, gankyrin (*PSMD10*), has been found to be associated with human cancers through diverse mechanisms (5-9). Gankyrin facilitates phosphorylation and degradation of *RB1* protein (pRb) *in vitro* and *in vivo* (10, 11), and activates the E2F family of transcription factors. In parallel, gankyrin competes with p16 for binding with cyclin-dependent kinase 4 (CDK4), which precludes the inhibition of p16 in CDK4-mediated phosphorylation of pRb, thus leading to cell cycle progression (8). Furthermore, gankyrin also functions in the degradation of the tumor suppressor protein p53 (12). By binding the ubiquitin ligase, murine double minute 2 (MDM2), gankyrin promotes p53 ubiquitylation and subsequent proteasomal degradation (13). Hence, gankyrin functions as a dual-negative regulator of the two most prominent tumor suppressor pathways: (i) INK4-CDK-pRb, and (ii) ARF-MDM2-p53. Moreover, gankyrin overexpression has been found to be a prevailing event in human hepatocellular carcinomas (HCCs) (6) and esophageal SCCs (ESCCs) (9), and emerging evidence shows that it could be one of the earliest molecular events occurring during epithelial cancer development. In a chemically induced rodent hepatocarcinogenesis model (6, 14), hypermethylation of *CDKN2A* and mutation of *TP53* appear during the later stages (HCC), whereas gankyrin is

*Both Authors contributed to this work equally.

Correspondence to: Dr. Junan Li, Division of Environmental Health Sciences, College of Public Health, The Ohio State University, Columbus, OH 43210, U.S.A. Tel: +1 6142924066, Fax: +1 6142924053, e-mail: li.225@osu.edu

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overexpressed much earlier, shortly after initial carcinogen exposure (liver fibrosis), preceding the loss of pRb (cirrhosis) and hepatocellular adenoma formation. In human HCCs, Tan *et al*. reported that the frequencies of gankyrin overexpression in Edmondson's grade I/II, III, and IV HCCs were 82%, 63%, and 22%, respectively (15). Consistently, 81% of HCCs at the low TNM stage and 35% of HCCs at the high TNM stage overexpressed gankyrin (16). In addition, a recent study on ESCCs (9) showed that gankyrin overexpression was positively correlated with lower survival rate, extent of the primary tumor, lymph node metastasis, distant lymph node metastasis and stage. These findings further indicate that gankyrin may be important during the development of the malignant potential in ESCC, and may play an important role in its progression (9). Thus, it would appear that gankyrin is one of few oncogenic proteins which negatively modulate both pRb and p53 pathways (11), and its aberrant overexpression could be an early initiating event in carcinogenesis. Since most oral carcinomas have inactivated pRb and p53 pathways either directly or indirectly (1-4), it is of interest to investigate the involvement of gankyrin during oral cancer development.

Here we report the first investigation into the incidence of altered gankyrin expression in oral cancer. Using quantitative real-time RT-PCR (qRT-PCR) and immunohistochemistry, we evaluated the expression of gankyrin in human oral lesions.

Materials and Methods

Cell culture and siRNA transfection. Seven oral cell lines were used, including a normal epithelial cell line (TE1177), one premalignant epithelial cell line (SCC-83-01-82), and five malignant cell lines (SCC-83-01-82CA, SCC9, SCC4, CAL27, and UM-SCC-22A) (17-20). SCC9, SCC4, and CAL27 were purchased from the American Type Culture Collection (Manassas, VA, USA); UM-SCC-22A was obtained from the University of Michigan; SCC-83-01-82, SCC-83-01-82CA, and TE1177 were kindly provided by Dr. S.M. D'Ambrosio of The Ohio State University (19, 20). All cells were cultured in Advanced DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS; Invitrogen) in a 90% relative humidity incubator at 37˚C supplied with 5% CO_2.

Synthetic siRNA targeting human gankyrin (26S proteasome non-ATPase regulatory subunit 10, *PSMD10*) (sc-72186; Santa Cruz Biotechnology, CA, USA) and control siRNA (sc-37007, Santa Cruz Biotechnology) were transfected into TE1177, SCC-83-01-82, and SCC-83-01-82CA cells using Oligofectamine (Invitrogen) in accordance with the manufacturer's protocol. Briefly, 6×10^6 cells (in a 10-cm diameter dish, 75% confluency) were transfected with siRNAs at a final concentration of 10 μM to knockdown more than 80% of *PSMD10* mRNA expression (data not shown). Upon transfection for 48 hours, the transfected cells were analyzed by flow cytometry to assess potential changes in the cell cycle distribution (propidium iodide staining) and apoptosis (Annexin-V-FLUOS staining) as previously described (21).

Patient sample procurement. All oral cancer and normal tissues were obtained from the Head and Neck Tumor Bank (J.C.L.) or Biorepository and Biospecimen Shared Resource at The Ohio State University Comprehensive Cancer Center following Institutional Review Board approved protocols. Two biospecimen sample sets were used for the retrospective studies: (i) Archival tissue blocks for 27 OSCC cases and 12 preneoplastic oral lesions, (ii) Total cellular RNA for 29 primary OSCCs, 2 recurrent OSCCs, 1 verrucous carcinoma, and 10 histologically normal oral tissues obtained from OSCC patients at the time of surgery (Table I).

Quantitative real-time RT-PCR. Total RNA was isolated from cells and tissues using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using PowerScript Reverse Transcriptase (BD Sciences, San Jose, CA, USA) as previously described (22). Real-time RT-PCR reactions were carried out using a SmartCycler system (Cepheid, Sunnyvale, CA, USA), and custom TaqMan assays designed using Primer Express 3.0 (Applied Biosystems, Carlsbad, CA, USA). Each reaction mixture included 200 μM dNTPs, 3 mM $MgSO_4$, 1.25 units of Taq polymerase (Agilent Technologies, Santa Clara, CA, USA), 1× Additive (0.2 mg/ml bovine serum albumin (BSA), 150 mM Trehalose, and 0.2% Tween 20), 0.1 volume of first-strand cDNA, 0.2 μM each primer, and 0.1 μM fluorogenic probe. The samples were amplified at 96˚C for 2 minutes, followed by 55 cycles of 30 seconds at 95˚C, 30 seconds at 60˚C, and 30 seconds at 68˚C. Human hypoxanthine phosphoribosyltransferase (*HPRT1*) was used for gene expression normalization. The primers and fluorogenic probes were as follows: *PSMD10*, 5'-TCCTCTTCATA TTGCGGCTT-3' (forward), 5'-CTTGAGCACCTTTTCCCAGAA-3' (reverse), and 5'-TET-TGG CCGGGATGAGATTGTAAAA GCC-TAMRA-3' (probe); *CDKN2A*, 5'-TGCCCAACGCACCGA-3' (forward), 5'-GGGCGC TGCCCATCA-3' (reverse), and 5'-TET-TAGTTACGGTCGG AGGCCG ATCCA-TAMRA-3' (probe); for *HPRT1*, 5'-CGGCT CCGTTATGGCG-3' (forward), 5'-GGTCA TAACCTGGTTCAT CATCAC-3' (reverse), and 5'-FAM-CGCAGCCCTGGCGTCGTGA-TAMRA-3' (probe). Each gene was amplified separately, and all experiments were performed in triplicate.

The expression levels of *PSMD10* and *CDKN2A* were normalized to that of *HPRT1*, as well as appropriate calibrators, and the relative gene expression level was determined using a comparative Ct method (23):

$\Delta\Delta Ct=(Ct_{PSMD10}-Ct_{HPRT1})_{tumor}-(Ct_{PSMD10}-Ct_{HPRT1})_{normal}$, or

$\Delta\Delta Ct=(Ct_{CDKN2A}-Ct_{HPRT1})_{tumor}-(Ct_{CDKN2A}-Ct_{HPRT1})_{normal}$

Relative gene expression=$2^{-\Delta\Delta Ct}$. A two-fold increase (≥2) or decrease (≤0.5) was considered significant for mRNA overexpression or down-regulation analysis, respectively (23).

Western blot. Total cellular proteins were extracted from oral normal and OSCC cells using the M-PER Mammalian Protein Extraction Reagent (ThermoScientific/Pierce, Rockford, IL, USA) and Western blotted as previously described (24). The primary and secondary antibodies were rabbit anti-human gankyrin antibody (PW8325; Enzo Life Sciences/BIOMOL, Plymouth Meeting, PA, USA) and peroxidase-conjugated rabbit anti-human IgG (ab6759; Abcam, Cambridge, MA, USA), respectively. Immunoblots were visualized by enhanced chemiluminescence using the SuperSignal West Pico kit (ThermoScientific/Pierce). Protein levels of β-actin were used for normalization.

Table I. *Demographics of the cohort of 32 OSCC patients and corresponding anatomic sites of tumor resection.*

	Sample ID	Gender	Age (years)	Race	Site	Comment
1	OSCC-001	Female	66	African-American	Tonsil	Primary
2	OSCC-004	Male	54	Caucasian	Larynx	Primary
3	OSCC-006	Male	62	Caucasian	Oral cavity	Primary
4	OSCC-007	Female	65	Caucasian	Tongue	Primary
5	OSCC-009	Male	42	Caucasian	Tongue	Primary
6	OSCC-010	Male	66	Caucasian	Oral cavity	Primary
7	OSCC-011	Male	66	Caucasian	Oral cavity	Primary
8	OSCC-012	Male	69	Caucasian	Oral cavity	Primary
9	OSCC-013	Female	45	Caucasian	Oral cavity	Primary
10	OSCC-014	Male	54	Caucasian	Oral cavity	Primary
11	OSCC-015	Female	47	African-American	Floor of mouth	Primary
12	OSCC-016	Male	47	Caucasian	Tonsil	Primary
13	OSCC-017	Female	75	Caucasian	Floor of mouth	Primary
14	OSCC-018	Male	70	Caucasian	Tongue	Primary
15	OSCC-019	Female	57	Caucasian	Tongue	Primary
16	OSCC-020	Male	42	Caucasian	Floor of mouth	Primary
17	OSCC-021	Male	66	Caucasian	Tongue	Primary
18	OSCC-022	Female	62	Caucasian	Floor of mouth	Primary
19	OSCC-024	Male	57	Caucasian	Tongue	Primary
20	OSCC-038	Female	65	African-American	Tonsil	Primary
21	OSCC-044	Male	59	Caucasian	Tongue	Primary
22	OSCC-054	Female	58	Caucasian	Oral cavity	Primary
23	OSCC-060	Male	51	Caucasian	Oral cavity	Primary
24	OSCC-023	Male	72	Caucasian	Floor of mouth	Primary
25	OSCC-066	Female	70	Caucasian	Tongue	Primary
26	OSCC-068	Female	48	Caucasian	Tongue	Primary
27	OSCC-070	Male	62	Caucasian	Tongue	Primary
28	OSCC-072	Male	61	Caucasian	Mandible	Primary
29	OSCC-074	Female	83	Caucasian	Maxilla	Recurrence
30	OSCC-075	Female	67	Caucasian	Palate	Verrucous
31	OSCC-077	Female	43	Caucasian	Maxilla	Primary
32	OSCC-064	ND	ND	ND	Maxilla	Recurrence

ND, Not documented.

Gankyrin immunohistochemical (IHC) staining and analysis. IHC detection of gankyrin was conducted on paraffin-embedded sections using standard avidin-biotin peroxidase procedure with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and an automated IHC System (BioGenex, San Ramon, CA, USA) as previously described (25). The percentage positive labeling index was calculated for each oral lesion stained utilizing Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA) analysis software. Histologically normal oral epithelial sections were utilized as controls.

Statistical analyses. All data in this study were statistically analyzed using R 2.9.2 software package. A two-sided *t*-test or χ^2 test at α=0.05 is regarded as significant.

Results

PSMD10/Gankyrin overexpression in OSCC cells. We first evaluated the expression of PSMD10/gankyrin in a 'normal' oral epithelium cell line (TE1177), a premalignant squamous epithelium cell line (SCC-83-01-82), and five malignant OSCC cell lines (SCC-83-01-82CA, SCC9, SCC4, CAL27, and UM-SCC-22A) (17-20) using qRT-PCR and immunoblotting. As shown in Figure 1A, the premalignant cells and all five OSCC cell lines exhibited significantly higher levels (2-8 fold) of *PSMD10* mRNA expression relative to TE1177 expression. Furthermore, results from Western blots (Figure 1B) demonstrated that the levels of gankyrin protein in these neoplastic oral cell lines were higher than that present in TE1177 cells. Therefore, gankyrin is overexpressed at both mRNA and protein levels in premalignant oral epithelial cells and malignant OSCC cell lines.

PSMD10/Gankyrin overexpression in human OSCC specimens. We then determined the expression of *PSMD10* mRNA in a retrospective cohort of 29 primary OSCCs, two recurrence OSCCs, and a verrucous carcinoma compared to the average expression in 10 representative histologically normal oral tissue specimens. As shown in Figure 2A, *PSMD10* mRNA was up-regulated in all 32 oral malignancies, with transcriptional

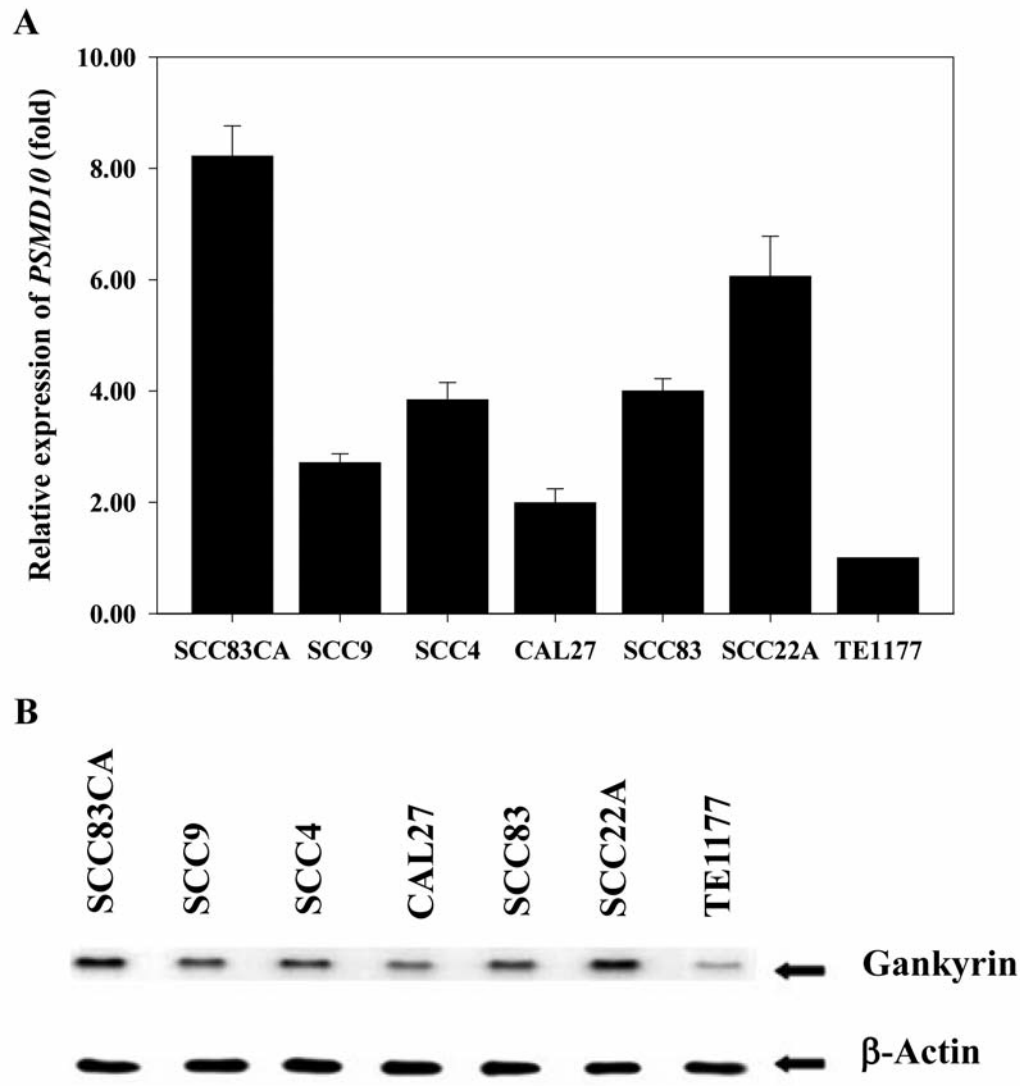

Figure 1. *Expression of PSMD10 mRNA and gankyrin protein in human OSCC cell lines. A: Quantitative real-time RT-PCR (qRT-PCR) determination of PSMD10 mRNA expression in oral cell lines. The constitutive metabolism gene HPRT1 was used for normalization. All experiments were performed in triplicate. Error bars represent standard deviations. B: Protein expression of gankyrin determined by Western immunoblot. The β-actin protein was used for normalization. In both A and B, TE1177 cells were used to represent the 'normal' state. The following cell lines were examined: SCC4, SCC9, CAL27, SCC-83-01-82 (SCC83), SCC-83-01-82CA (SCC83CA), UM-SCC-22A (SCC22A), and TE1177.*

overexpression ranging from 0.3- to 90-fold. While it remains unknown what biological level of overexpression generates a significant physiological event for *PSMD10*, using a more rigid criterion for mRNA overexpression (≥2-fold change) (23) demonstrated that 84% of these OSCCs (27/32) overexpressed *PSMD10* mRNA. It is worth noting that none of these clinical specimens demonstrated a reduction in *PSMD10* expression. Since down-regulation of *CDKN2A* is one of the most prevalent molecular alterations found in oral cancer (26), we also assessed the expression of *CDKN2A* mRNA in these clinical samples (Figure 2B). Consistent with our previous studies (25) and the current knowledge base, *CDKN2A* mRNA was poorly expressed in 65.6% of the specimens (21/32), expressed at normal levels in 25.0% of the samples (8/32), and overexpressed in 9.4% of the specimens (3/32). These findings support existing studies and demonstrate that down-regulation of *CDKN2A* expression remains one of the major events during oral carcinogenesis (26). However, no significant correlation between *PSMD10* overexpression and *CDKN2A* down-regulation (p=0.35) was demonstrated in these clinical samples, suggesting that these two transcriptional events may be independent biomarkers of oral malignancy in this patient cohort.

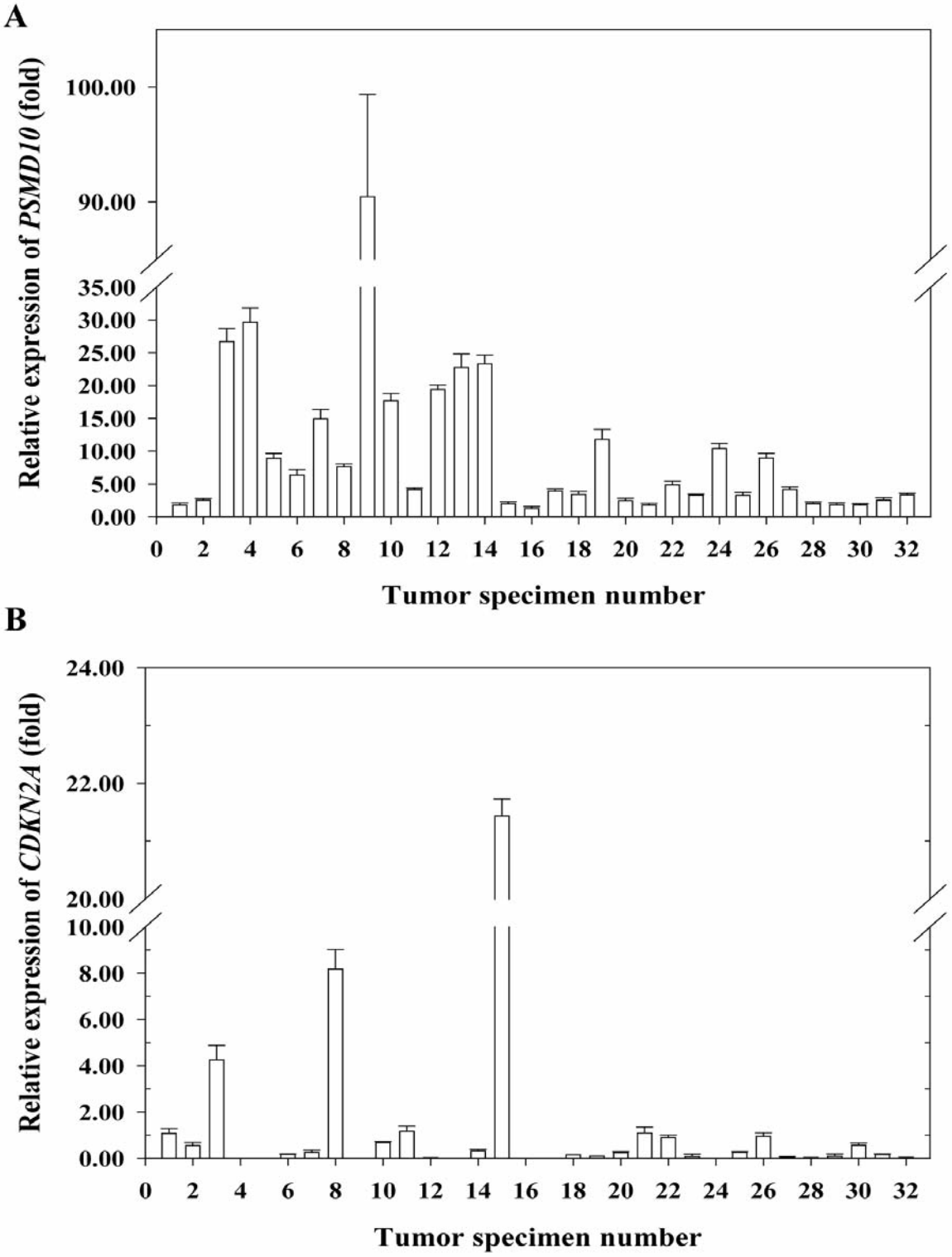

Figure 2. *Expression of PSMD10 and CDKN2A mRNA in human malignant oral lesions. A total of 32 OSCC specimens were analyzed using real-time qRT-PCR. A pooled sample from 10 representative histologically normal oral tissues was used for normalization. All experiments were performed in triplicate. Error bars represent standard deviations. A: PSMD10 mRNA expression in malignant oral tissues. Values (y-axis) represent fold-changes calibrated to pooled normal tissue expression levels. B: CDKN2A mRNA expression in malignant oral tissues. Values (y-axis) represent fold-changes calibrated to pooled normal tissue expression levels. See Table I for tumor designations.*

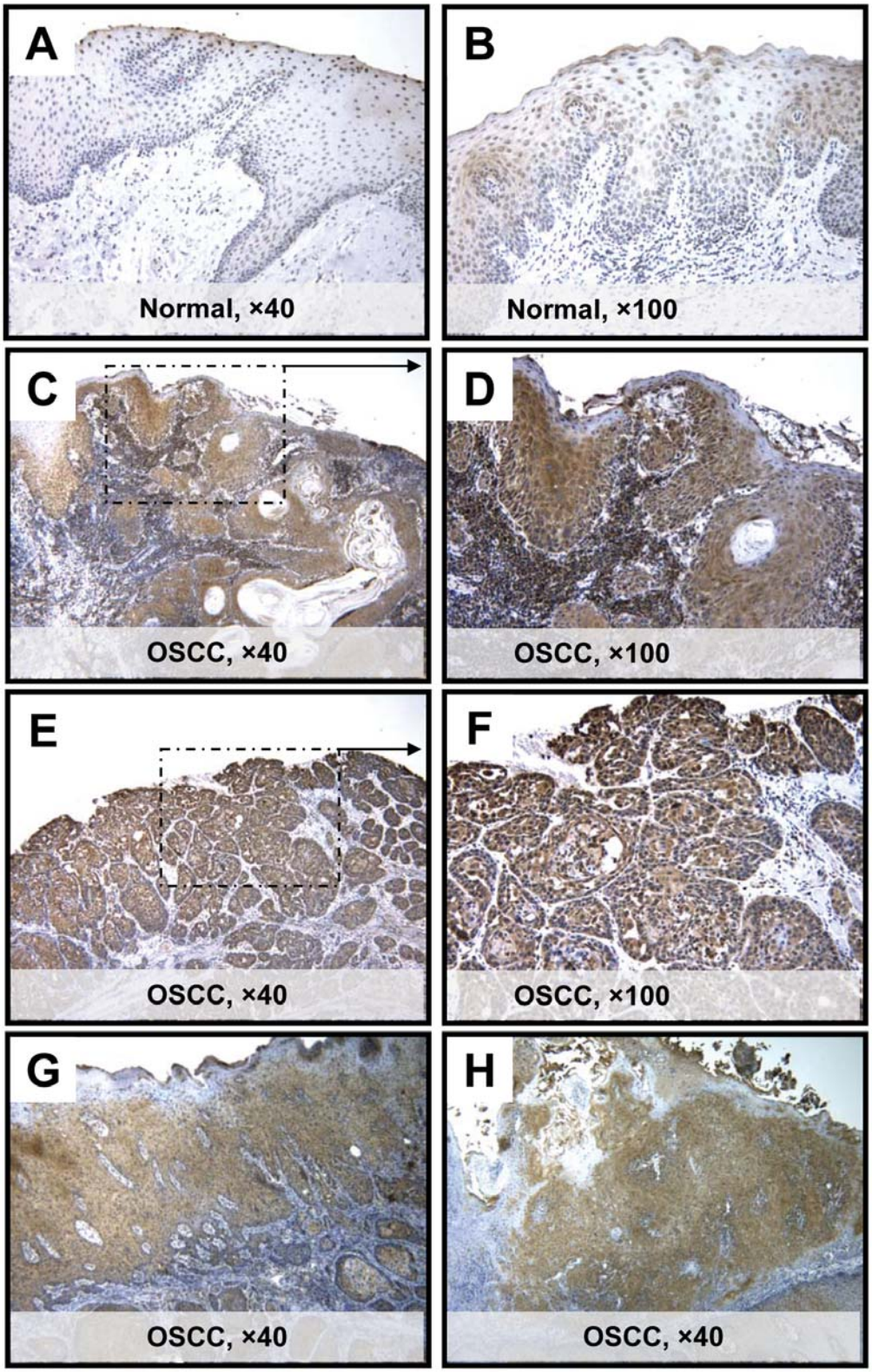

Figure 3. *Gankyrin protein expression in human malignant oral tissues. Immunohistochemical analysis of gankyrin protein expression in representative human oral normal and malignant tissues. A: Normal oral epithelium, ×40 magnification. B: Normal oral epithelium, ×100 magnification. C: OSCC, ×40 magnification. D: OSCC from panel C, ×100 magnification. E: OSCC, ×40 magnification. F: OSCC from panel D, ×100 magnification. G: OSCC, ×40 magnification. H: OSCC, ×40 magnification.*

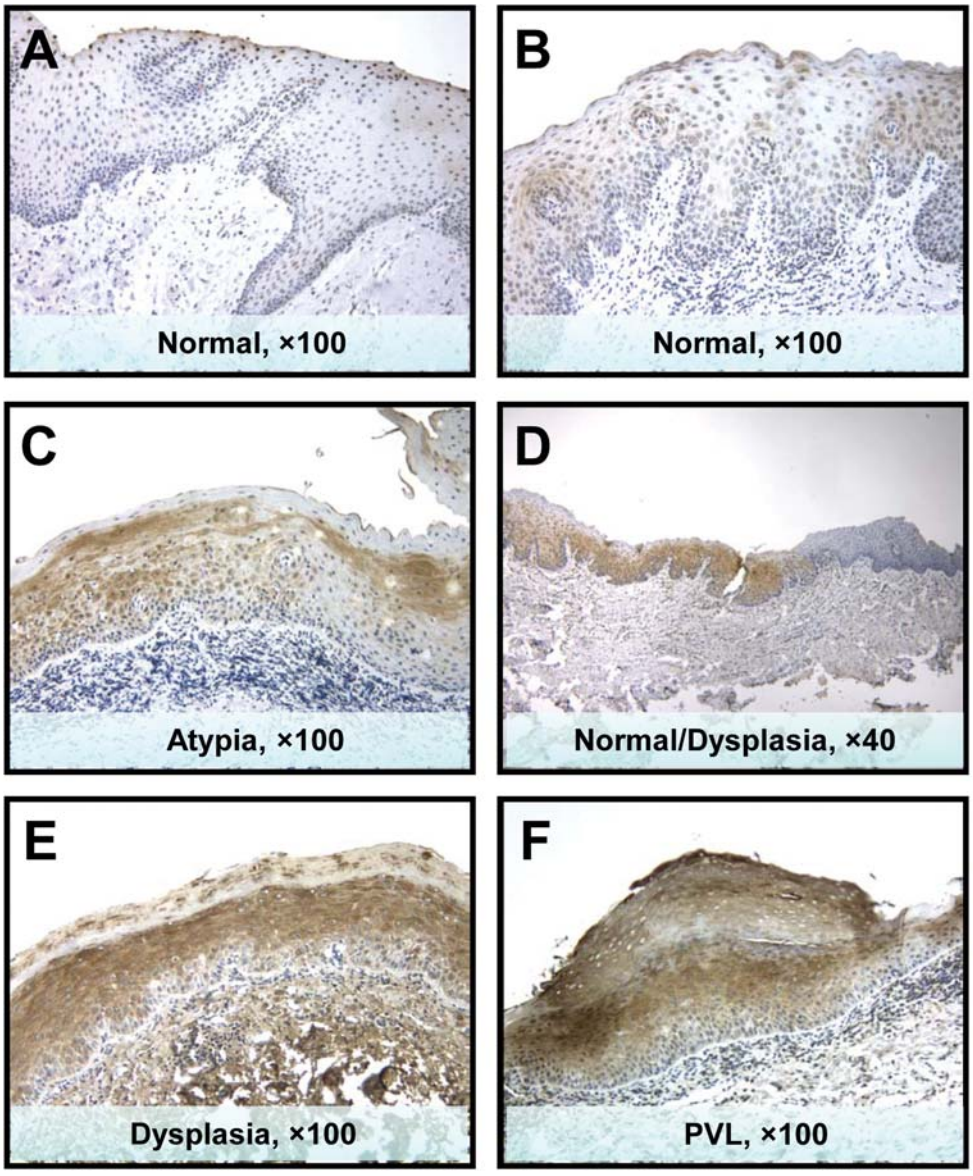

Figure 4. *Gankyrin protein expression in human premalignant oral lesions. Immunohistochemical analysis of gankyrin protein expression in representative human premalignant oral tissues. A: Normal oral epithelium I, ×100 magnification. B: Normal oral epithelium II, ×100 magnification. C: Oral epithelium presenting with atypia, ×100 magnification. D: Normal and mild/low-grade dysplasia, ×40 magnification. E: Mild/low-grade dysplasia, ×100 magnification. F: Proliferative verrucous leukoplakia (PVL), ×100 magnification.*

We further examined the presence of gankyrin protein using IHC in a distinct retrospective OSCC patient cohort (n=27) for which only archival paraffin tissue blocks were available (24). Figure 3 shows representative IHC results for gankyrin protein expression in normal oral and OSCC tissues. Mild staining is apparent in the normal tissue (Figure 3A and 3B), which is consistent with one of the basal activity roles of gankyrin as a transient subunit of the proteasome complex (11). In contrast, the majority of OSCCs (21/27, 71%) exhibited clear overexpression of gankyrin protein (Figure 3C-H). The difference in incidence of overexpression of *PSMD10* mRNA and gankyrin protein (84% *vs*. 71%) is not statistically significant (p=0.75), providing support for a possible simple mechanism linking transcription and translation. These results demonstrate that overexpression of gankyrin protein occurs frequently during oral carcinogenesis and may be the result of deregulation at the transcriptional level (*i.e*. overexpression of *PSMD10* mRNA) resulting in elevated gankyrin protein levels.

Gankyrin overexpression in preneoplastic oral lesions. Since gankyrin overexpression has been reported to occur during the earliest stages of hepatocellular carcinogenesis, prior to prominent cancer-promoting events such as pRb degradation and hypermethylation of *CDKN2A* and *TP53* (6, 14) we postulated that activation of proto-oncogenic gankyrin could be an early event in the development of oral cancer. To address the temporal alteration of gankyrin expression during oral malignant neogenesis, we examined the presence of gankyrin protein in oral tissues at different stages of cancer development using IHC. Figure 4 shows gankyrin protein expression in representative normal oral tissue (Figure 4A and 4B), mild/low-grade dysplasia (Figure 4C), severe/high-grade dysplasia (Figure 4D and 4E), and proliferative verrucous leukoplakia (PVL) (Figure 4F). Mild staining was apparent in the normal tissue, which is consistent with the constitutive function of gankyrin as a component of the 26S proteasome complex. In contrast, gankyrin protein was intensely expressed in the premalignant mild/low-grade dysplasia (Figure 4C), severe/high-grade dysplasia (Figure 4D and 4E), and PVL lesion (Figure 4F). In this premalignant oral tissue group we demonstrate gankyrin protein overexpression with respect to normal oral epithelium in 6/12 of the cases. These results provide support for a role of gankyrin protein overexpression early during oral carcinogenesis, and establish a foundation for examining gankyrin as one of the earliest events in oral epithelial cell deregulation.

Gankyrin promotes the proliferation of premalignant and malignant oral cancer cells in vitro. To evaluate the effect of gankyrin expression in oral cancer cells, we transfected three oral cell lines, TE1177 (normal), SCC-83-01-82 (premalignant), SCC-83-01-82CA (malignant) with siRNA targeting human gankyrin (sc-72186; Santa Cruz Biotechnology) and analyzed changes in cell cycle distribution using flow cytometry. The results are listed in Table II. As mentioned earlier (Figure 1), gankyrin was overexpressed at both mRNA and protein levels in SCC-83-01-82 and SCC-83-01-82CA cells (in comparison with TE1177). In the absence of any treatment, about 72.0% of TE1177 cells were in the G_0/G_1 phase, compared to 51.2% of SCC-83-01-82 and 49.8% of SCC-83-01-82CA cells. While gankyrin-targeting siRNA treatment did not bring about any significant changes in the cell cycle distribution of TE1177 cells, siRNA-mediated down-regulation of gankyrin expression in premalignant SCC-83-01-82 cells led to a significant increase of cells in the G_0/G_1 phase (51.2% *vs.* 58.7%, $p<0.05$). Such increase in the G_0/G_1 population was even greater in siRNA-treated malignant SCC-83-01-82CA cells (49.8% *vs.* 62.6%, $p<0.01$). In contrast, control siRNA did not bring about significant changes in the cell cycle distribution of any of these oral cell lines. Additionally, no significant changes in apoptosis were observed in any of these cell lines upon transfection of gankyrin-targeting siRNA (data not shown). Hence, down-regulation of gankyrin expression in premaligant and malignant oral cancer cells primarily prohibited cell proliferation. This finding is consistent with previous studies showing that gankyrin enhances the growth and proliferation, not apoptosis, of pancreatic and lung cancer cells (21, 27). Taken together, these results suggest that aberrant gankyrin expression contributes to human cancer progression through promoting cell proliferation.

Table II. *siRNA-mediated down-regulation of gankyrin in oral cells*.

Oral cell line	siRNA	Cell cycle distribution (%)		
		G_0/G_1	S	G_2/M
TE1177	None	72.0±0.5	13.5±1.1	14.5±0.7
'normal'	Control siRNA	72.7±1.5	12.9±0.8	14.4±1.7
	PSMD10 siRNA	74.8±3.5	13.9±2.2	11.3±1.4
SCC-83-	None	51.2±0.8	29.5±1.2	18.3±1.6
01-82	Control siRNA	50.7±2.1	30.6±1.4	18.7±2.3
premalignant	*PSMD10* siRNA	58.7±2.4	25.5±2.0	15.8±2.5
SCC-83-01-	None	49.8±0.4	33.2±1.7	17.0±2.2
82CA	Control siRNA	51.8±3.3	31.8±2.8	16.4±1.4
malignant	*PSMD10* siRNA	62.6±3.7	25.3±1.3	12.1±2.1

All data were from three independent experiments.

Discussion

The development of oral cancer proceeds through well-defined sequential stages from normal epithelium, through atypic and hypeplastic phenotypes, mild, moderate, and severe dysplasias, and culminates in OSCC with possible metastatic disease (3, 25). While there are established genetic landmarks during oral cancer progression, the earliest initiating events remain less well characterized and continue to be of particular interest in cancer prognostic, diagnostic, and prevention strategies. Our current studies provide significant foundational evidence for an early role of oncogenic gankyrin in oral cancer development, and provide support for examining the potential of gankyrin as an early biomarker of oral cancer progression.

Aberrant gankyrin expression at the transcriptional and translational levels represents a prevalent molecular event during oral carcinogenesis. Similar to the observations of gankyrin overexpression in human and rat HCC and human ESCCs, we observed significant overexpression of *PSMD10* mRNA in 6/6 of OSCC cell lines and 27/32 (84%) of malignant oral lesions. The striking incidence of *PSMD10* mRNA overexpression is recapitulated by our studies on

gankyrin protein expression in OSCC cell lines and tumor tissues. Mimicking their *PSMD10* overexpression, all six OSCC cell lines overexpressed gankyrin protein. Similarly, 71% of OSCC tissues had intense staining of gankyrin protein compared to patient-matched normal oral tissues (25). Furthermore, activation of gankyrin (overexpression) occurs during the early stages of oral cancer development. In the established rat model of chemically induced HCC, distinct molecular events occur sequentially during the multi-step process of hepatocellular carcinogenesis. These initiating and promoting actions include gankyrin overexpression, pRb degradation, and hypermethylation of *CDKN2A* and *TP53* (6, 14). In our current studies, gankyrin also maintains a prominent early role in the multistep process of oral carcinogenesis, with 50% of oral preneoplastic lesions demonstrating a higher level of gankyrin protein expression compared to their normal tissue counterparts.

While alterations in *CDKN2A* mRNA and p16 protein represent early biomarkers of oral cancer development and are components of a common cell cycle regulatory pathway, our current studies do not demonstrate a direct association between gankyrin overexpression and the functional status of the *CDKN2A* gene in this OSCC patient cohort. While both up-regulation of *PSMD10*/gankyrin and down-regulation of *CDKN2A* occur in these retrospective cohorts, it is possible that these events represent independent biomarkers of oral carcinogenic progression. The genetic status of *CDKN2A* varies among the gankyrin overexpressing-OSCC cell lines. UM-SCC-22A cells display a homozygous *CDKN2A* deletion, while SCC9 cells have a point mutation in the *CDKN2A* promoter, and CAL27 cells lack functional p16 due to unknown mechanisms (20). Conversely, SCC4, SCC83, and SCC83CA cells have an intact *CDKN2A* gene (17-20). Since both overexpression of gankyrin and loss of p16 expression contribute to control of cell cycle progression through altering the CDK4-mediated phosphorylation of pRb protein (8), it is of interest whether it is necessary to have an additional mechanism of pRb inactivation (gankyrin up-regulation) in a pathway that already exhibits a prevalent inactivating event (loss of functional p16). Support for the value of gankyrin overexpression as an important biomarker during oral carcinogenesis comes from the following observations: (i) Gankyrin overexpression occurs during the earliest stages of oral cancer progression, possibly in advance of the known biomarker p16 deregulation; (ii) Previous studies have shown that approximately 40% of OSCCs contain intact *CDKN2A* or maintain functional p16 despite the presence of missense mutations (26). In these cases, functional p16 would remain subject to gankyrin-mediated cell cycle interference, and initiated premalignant cells could continue through the cell cycle (8); (iii) Gankyrin functions through distinct mechanisms and regulatory pathways, including the cell cycle *via* CDK4 interference, MDM2-mediated protein degradation of pRb and p53 *via* the proteasome complex, and p53-mediated apoptotic suppression (5-9). Early alterations in *PSMD10*/gankyrin can generate diverse multifactorial consequences independently capable of modulating cell growth and carcinogenic progression.

In conclusion, our results demonstrate for the first time that gankyrin is frequently overexpressed in premalignant and malignant oral tissues, further suggesting that activation of gankyrin is an early event during oral carcinogenesis. The sensitivity and specificity of gankyrin as a putative oral cancer biomarker will need to be evaluated in larger populations and prospective studies. The specific environmental or genotoxic exposures responsible for activation of gankyrin in oral cancer are unknown; however, it is known that tobacco smoke is a significant risk factor for both OSCC and HCC. Given the striking incidence of gankyrin overexpression in HCCs and the common risk factor of smoking, it is possible that tobacco carcinogens could activate the proto-oncogenic *PSMD10* gene in oral epithelial cells in the field of cancerization, and provide the metabolic advantage necessary to initiate and promote deregulated cell growth. These findings provide novel insights into early molecular markers of oral cancer development and emphasize the need for additional studies on the potential of gankyrin as a biomarker, therapeutic, or chemopreventive target.

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References

1 Reed AL, Califano J, Cairns P, Westra WH, Jones RM, Koch W, Ahrendt S, Eby Y, Sewell D, Nawroz H, Bartek J and Sidransky D: High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res *56*: 3630-3633, 1996.

2 Rocco JW and Sidransky D: p16(MTS-1/CDKN2/INK4a) in cancer progression. Exp Cell Res *264*: 42-55, 2001.

3 Kresty LA, Mallery SR, Knobloch TJ, Song H, Lloyd M, Casto BC and Weghorst CM: Alterations of p16(INK4a) and p14(ARF) in patients with severe oral epithelial dysplasia. Cancer Res *62*: 5295-5300, 2002.

4 Bosch FX, Ritter D, Enders C, Flechtenmacher C, Abel U, Dletz A, Hergenhahn M and Weidauer H: Head and neck tumor sites differ in prevalence and spectrum of p53 alterations but these have limited prognostic value. Int J Cancer *111*: 530-538, 2004.

5 Higashitsuji H, Itoh K, Nagao T, Dawson S, Nonoguchi K, Kido T, Mayer RJ, Arii S and Fujita J: Reduced stability of retinoblastoma protein by gankyrin, an oncogenic ankyrin-repeat protein overexpressed in hepatomas. Nat Med *6*: 96-99, 2000.

6 Park TJ, Kim HS, Byun KH, Jang JJ, Lee YS and Lim IK: Sequential changes in hepatocarcinogenesis induced by diethylnitrosamine plus thioacetamide in Fischer 344 rats: Induction of gankyrin expression in liver fibrosis, pRB degradation in cirrhosis, and methylation of $p16^{INK4a}$ exon 1 in hepatocellular carcinoma. Mol Carcinogenesis *30*: 138-150, 2001.

7 Dawson S, Apcher S, Mee M, Higashitsuji H, Baker R, Uhle S, Dubiel W, Fujita J and Mayer RJ: Gankyrin is an ankyrin-repeat oncoprotein that interacts with CDK4 kinase and the S6 ATPase of the 26S proteasome. J Biol Chem *277*: 10893-10902, 2002.

8 Li J and Tsai MD: Novel insights into the INK4-CDK4/6-Rb pathway: counter action of gankyrin against INK4 proteins regulates the CDK4-mediated phosphorylation of Rb. Biochemistry *41*: 3977-3983, 2002.

9 Oritz CM, Ito T, Tanaka E, Tsunoda S, Nagayama S, Sakai Y, Higashitsuji H, Fujita J and Shimada Y: Gankyrin oncoprotein overexpression as a critical factor for tumor growth in human esophageal squamous cell carcinoma and its clinical significance. Int J Cancer *122*: 325-332, 2008.

10 Ying HQ and Xiao ZJ: Targeting retinoblastoma protein for degradation by proteasomes. Cell cycle *5*: 506-508, 2006.

11 Mayer RJ and Fujita J: Gankyrin, the 26S proteasome, the cell cycle and cancer. Biochem Soc Trans *34*: 746-748, 2006.

12 Higashitsuji H, Higashitsuji H, Itoh K, Sakurai T, Nagao T, Sumitomo Y, Masuda T, Dawson S, Shimada Y, Mayer RJ and Fujita J: The oncoprotein gankyrin binds to MDM2/HDM2 enhancing ubiquitylation and degradation of p53. Cancer Cell *8*: 75-87, 2006.

13 Higashitsuji H, Liu Y, Mayer RJ and Fujita J: The oncoprotein gankyrin negatively regulates both p53 and RB by enhancing proteasomal degradation. Cell cycle *4*: 1335-1337, 2005.

14 Lim K: Spectrum of molecular changes during hepato-carcinogenesis induced by DEN and other chemicals in Fisher 344 male rats. Mech Ageing Dev *124*: 1665-1680, 2002.

15 Tan L, Fu XY, Liu SQ, Li HH, Hong Y, Wu MC, and Wang HY: Expression of $p28^{GANK}$ and its correlation with RB in human hepatocellular carcinoma. Liver Int *25*: 667-676, 2005.

16 Umemura A, Itoh Y, Itoh K, Yamaguchi T, Nakajima T, Higashitsuji H, Onoue H, Fukumoto M, Okanoue T and Fujita J: Association of gankyrun protein expression with early clinical stages and insulin-like growth factor-binding protein 5 expression in human hepatocellular carcinoma. Hepatology *47*: 493-502, 2008.

17 Li J, Muscarella P, Joo SH, Knobloch TJ, Melvin ES, Weghorst CM and Tsai MD: Dissection of CDK4-binding and transactivation activities of $p34^{SEI-1}$ and comparisons between functions of $p34^{SEI-1}$ and $p16^{INK4A}$. Biochemistry *44*: 13246-13256, 2005.

18 Timmermann S, Hinds PW, and Munger K: Re-expression of endogenous $p16^{INK4a}$ in oral squamous cell carcinoma lines by 5-aza-2'-deoxycytidine treatment induces a senescence-like state. Oncogene *17*: 3445-3453, 1998.

19 Han C, Ding H, Casto B, Stoner GD and D'Ambrosio SM: Inhibition of the growth of premalignant and malignant human oral cell lines by extracts and components of black raspberries. Nutrition and Cancer *51*: 207-217, 2005.

20 D'Ambrosio SM, D'Ambrosio REG, Milo GE, Casto BC, Kelloff GJ and Steele VE: Differential growth responses of normal, premalignant, and malignant human oral epithelial cells by chemopreventive agents. Anticancer Res *20*: 2273-2280, 2000.

21 Meng Y, He L, Guo X, Tang S, Zhao X, Du R, Jin J, Bi Q, Li H, Nie Y, Liu J and Fan D: Gankyrin promotes the proliferation of human pancreatic cancer. Cancer Lett *297*: 9-17, 2010.

22 Yip H, Chopra R, Chakrabarti R, Veena MS, Ramamurthy B, Srivatsan ES and Wang MB: Cisplatin-induced growth arrest of head and neck cancer cells correlates with increased expression of p16 and p53. Arch Otolaryngol Head Neck Surg *132*: 317-326, 2006.

23 Kanellou P, Zaravinos A, Zioga M, Stratigos A, Baritaki S, Soufla G, Zoras O and Spandidos DA: Genomic instability, mutations and expression analysis of the tumor suppressor $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$ and p53 in actinic keratosis. Cancer Lett *264*: 145-161, 2008.

24 Li J, Zhu J, W Melvin S, Bekaii-Saab TS, Chen CS and Muscarella P: A structurally optimized Celecoxib derivative inhibits human pancreatic cancer cell growth. J Gastroint Surg *10*: 206-213, 2006.

25 Kresty LA, Mallery SR, Knobloch TJ, Li J, Lioyd M, Casto BC and Weghorst CM: Frequent alterations of $p16^{INK4a}$ and $p14^{ARF}$ in oral proliferative verrucous leukoplakia. Cancer Epidemiol Biomarkers Prev *17*: 3179-3187, 2008.

26 Lang JC, Borchers J, Danahey D, Smith S, Stover DG, Agrawal A, Malone JP, Schuller DE, Weghorst CM, Holinga AJ, Lingam K, Patel CR and Esham B: Mutational status of overexpressed p16 in head and neck cancer: evidence for germline mutation of $p16/p14^{ARF}$. Int J Oncol *21*: 401-408, 2002

27 Man JH, Liang B, Gu YX, Zhou T, Li AL, Li T, Jin BF, Bai B, Zhang HY, Zhang WN, Li WH, Gong WL, Li HY and Zhang XM: Gankyrin plays an essential role in Ras-induced tumorigenesis through regulation of the RhoA/ROCK pathway in mammalian cells. J Clin Invest *120*: 2829-2841, 2010.

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