ORIGINAL ARTICLE

Chronic Periodontitis–Human Papillomavirus Synergy in Base of Tongue Cancers

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Objective: To assess whether chronic periodontitis history predicts human papillomavirus (HPV) status in patients with base of tongue cancers.

Design: Case-control study using existing patient data.

Setting: Roswell Park Cancer Institute.

Patients: Thirty patients newly diagnosed with base of tongue squamous cell carcinoma between 1999 and 2005 for whom both tumor samples and periodontal records were available. Patients younger than 21 years, edentulous, immunocompromised, and those with a history of cancer were excluded. Periodontitis history was assessed on the basis of alveolar bone loss (in millimeters) from panoramic radiographs by one examiner who was blinded to cancer status.

Main Outcome Measure: HPV-16 and HPV-18 DNA were identified on paraffin-embedded tumor samples by polymerase chain reaction. Multiple logistic regression was used to estimate odds ratios and 95% confidence intervals.

Results: The prevalence of tumors positive for HPV-16 DNA was 21 of 30 (70%). None of the samples were positive for HPV-18 DNA. Compared with participants with HPV-negative tumors, patients with HPV-positive tumors had significantly higher mean alveolar bone loss (3.90 mm vs 2.85 mm, $P=.01$). After adjustment for age at diagnosis, sex, race/ethnicity, alcohol use, smoking status, and number of missing teeth, every millimeter of alveolar bone loss was associated with an approximately 4-fold (odds ratio, 3.96; 95% confidence interval, 1.18-13.36) increased risk of HPV-positive tumor status. Number of missing teeth was not associated with tumor HPV status (odds ratio, 0.95; 95% confidence interval, 0.74-1.21).

Conclusions: Chronic periodontitis may be a significant factor in the natural history of HPV infection in patients with base of tongue cancers. Additional confirmation in larger studies is required.

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IT IS NOW RECOGNIZED THAT HUman papillomavirus (HPV) may be an additional independent risk factor for a subset of head and neck cancers (HNCs).[1,2] However, most HPV infections are cleared rapidly and do not cause malignancy. Persistence of HPV infection is a central risk factor for carcinogenesis.[2] Therefore, understanding the factors for the persistence of oral HPV is critical to facilitate effective prevention of HNCs. Coinfections

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in the cervix with particular bacteria such as *Chlamydia trachomatis* and chronic cervicitis were shown to act synergistically with HPV to increase cervical cancer risk.[3] In addition, increased levels of inflammatory cytokines, such as interleukins 1 and 6 and tumor necrosis factor α, were shown to modulate HPV gene expression and proliferation.[4,5] In a recent study, a nonsteroidal anti-inflammatory drug induced specific degradation of the HPV oncoprotein E7 and caused growth arrest and apoptosis in cervical carcinoma cells.[6]

Periodontitis is a chronic oral infection caused by inflammatory reactions to gram-negative anaerobic bacteria in the dental plaque.[7] It leads to irreversible destruction of tissues around teeth, clinically detectable as periodontal pockets and alveolar bone loss (ABL). Periodontitis results in a continuous release of bacterial toxins and inflammatory markers into saliva and, to a lesser degree, into the bloodstream.[8] Human papillomavirus infects exclusively the basal cells of the epithelium. It gains access through abrasions or through normal exposure of parabasal cells, such as in the transformation zone of the uterine cervix. In addition, replication of virus is closely associated with proliferation of the basal and parabasal cells.[9] The periodontal pocket is composed of

stratified squamous epithelium and is characterized by continuous epithelial proliferation, migration, reteridge formation, and ulcerations, providing ample opportunity for initial HPV infection and its persistence. Integrin $\alpha_6\beta_4$ and syndecan-1, candidate receptors for HPV, are expressed during wound healing and are present in the periodontal pocket epithelium.[10]

It was also suggested that periodontal pockets may serve as a reservoir for latent HPV.[10,11] Latent state would require the presence of infected cells that fail to differentiate.[9] The junctional epithelium at the bottom of the periodontal pocket appears to fully serve the cellular functions required by HPV. The junctional epithelium is composed of basal and suprabasal cell layers only and has a very high turnover rate. The basal cells are exfoliated through the gingival crevice before differentiation occurs.[10] Frequent occurrence of gingival papillomas and condylomas in human immunodeficiency virus (HIV)–positive participants and in patients with organ transplants receiving cyclosporine treatment could be explained by reactivation of latent HPV.[12]

In a recent study funded by the National Institutes of Health, we found an independent association between chronic periodontitis and the risk of tongue cancer.[13] Based on these observations, we conducted the present study to test the hypothesis that chronic periodontitis is a predictor of the tumor HPV status in patients with base of tongue squamous cell carcinoma.

METHODS

STUDY DESIGN AND POPULATION

We performed a hospital-based case-control study. The study population was derived from the patient population of the Department of Dentistry and Maxillofacial Prosthetics (DMFP), Roswell Park Cancer Institute. All patients newly diagnosed with primary base of tongue squamous cell carcinoma (*International Classification of Diseases for Oncology*, 3rd edition, code C01.0)[14] between June 15, 1999, and January 10, 2005, who had tissue samples available through Pathology Department archives were included. Patients younger than 21 years, edentulous, immunocompromised, and those with a history of cancer were excluded. All patients with HNC who receive radiotherapy and/or chemotherapy are admitted to the DMFP before the initiation of their cancer treatment. From a total of 47 newly diagnosed patients with base of tongue cancer who were diagnosed during the study period, 33 were admitted to the DMFP. Of those 33 patients, 3 were edentulous. Therefore, 30 patients had both periodontal and pathology data available (26 men and 4 women). The study protocol was approved by the institutional review boards of the Roswell Park Cancer Institute and the State University of New York at Buffalo.

ASSESSMENT OF PERIODONTITIS

History of periodontitis was assessed quantitatively on the basis of ABL from panoramic radiographs that were taken at admission before the cancer treatment was initiated. Radiographs were taken with a software-controlled orthopantomograph (model OP100; Instrumentarium Imaging, Tuusula, Finland) using standard exposure time (17.6 seconds) and settings (70 kVp and 12 mA). Patient positioning was also standardized with the help of head, temple, and chin supports; bite fork; occlusion adjustment keys; and positioning lights.

Alveolar bone loss is an established measure of periodontitis history.[15] It is an outcome measure and quantifies cumulative vertical bone loss as a result of inflammation. The distance between the cementoenamel junction and the bone crest in a line parallel to the long axis of the tooth is measured in millimeters. Measurements were performed on mesial and distal surfaces of all natural teeth using an operator-interactive program on digitized radiographic images by a trained periodontist. Typically, ABL less than 2 mm is considered to represent normal or healthy periodontium. Accuracy and reliability of this method have been previously established.[15] In the present study, all ABL measurements were performed by one examiner who was blinded to HPV status. Duplicate ABL measurements were performed on 5 randomly selected study participants with a 3-day interval to establish intraexaminer reliability. The mean (SD) differences of the duplicate measurements was 0.22 (0.41) mm.

DETECTION OF TUMOR HPV DNA

The presence or absence of HPV DNA in tumor tissue was determined by polymerase chain reaction (PCR). All specimens were reviewed by a pathologist to confirm the presence of tumor on slides. DNA extraction and PCR amplification are performed in separate laboratories to reduce contamination.

DNA EXTRACTION

Three 10-µm sections were cut from paraffin-embedded tumor sections as identified in the Pathology Department to ensure adequate isolated DNA from tumor tissue. DNA was extracted from tumor samples using previously described procedures.[16] Microtome blades were changed between each embedded block to prevent cross-contamination among specimens. Paraffin was removed through xylene treatment, and the specimen was rehydrated in a decreasing ethanol gradient. An overnight digestion at 60°C in a cocktail containing 50mM potassium chloride, 1.5mM magnesium chloride, 10mM TRIS hydrochloride, 0.5% polyethylene glycol sorbitan monolaurate [TWEEN 20; Sigma-Aldrich Corp, St Louis, Missouri], and 200 µg/mL proteinase K was followed by RNase A (100 µg/mL) digestion, phenol/chloroform extraction, and ethanol precipitation. This procedure produces high-molecular-weight DNA suitable for PCR analyses.

HPV AMPLIFICATION BY PCR

Purified DNA (100 ng) served as a template in each of 3 separate 25-µL reactions. Reactions 1 and 2 contained 0.25mM forward and reverse type-specific primers for the E6 regions of HPV-16 and HPV-18, 1.5mM magnesium chloride, 2.0mM deoxynucleotide triphosphates, and 1 U of *Taq* DNA Polymerase (Invitrogen, Carlsbad, California) in a 1× amplification buffer. The forward and reverse primers for these reactions were HPV-16 forward 5′-ATTAGTGAGTATAGACATTA-3′; HPV-16 reverse 5′-GGCTTTTGACAGTTAATACA-3′; HPV-18 forward 5′-ATTAGAGAATTAAGACATTA-3′; and HPV-18 reverse 5′-GGTTTCTGGCACCGCAGGCA-3′. Reaction 3, a control that amplifies a DNA fragment from the housekeeping gene β-*globin*, differs from reactions 1 and 2 only in the primers added and in that it contains 2.5mM magnesium chloride. The forward and reverse primers for this reaction were β-*globin* forward 5′-CAACTTCATCCACGTTACCC-3′ and β-*globin* reverse 5′-GAAGAGCCAAGGACAGGTAC-3′. All PCRs were assembled in a laminar flow sterile cabinet dedicated to that

purpose. Triplicate amplifications were performed in a thermocycler (model PTC 100; MJ Research, Inc, Waltham, Massachusetts) as follows: initial denaturation at 95°C for 7 minutes followed by 40 cycles at 95°C for 45 seconds (denaturation), 52°C for 1 minute (annealing), 72°C for 1 minute (extension), and a 5-minute final extension at 72°C. Plasmids containing the E6 region of HPV-16 or HPV-18 were used as positive controls for PCR amplification. A mock reaction containing no DNA was used as a negative control.

HPV AMPLIMER DETECTION

Of each PCR, 5 µL was resolved by electrophoresis on a 3% LE (low electroendosmosis) agarose gel containing 1 µg/mL ethidium bromide. The presence of a 109–base pair fragment in either of the first 2 reactions confirmed the presence of one of the HPV isotypes in the sample, while a 268–base pair β-*globin* fragment in reaction 3 established the ability of the DNA to be amplified in the absence of either HPV band. Samples that were negative for β-*globin* were considered to be of insufficient quality for analysis and were excluded. Amplified DNA was visualized by ultraviolet transillumination. Tumor-derived samples of 10 to 100 ng of genomic DNA containing approximately 100 molecules of viral DNA routinely score positive in PCR assays. Primer design allows for viral type-specific amplification. Tumors that are positive for amplification of the HPV-16 E6 region do not amplify with primers for the highly homologous region of HPV-18.

STATISTICAL ANALYSES

Means, standard deviations, frequencies, and proportions of available relevant variables were used to describe the study population. Selection and definition of covariates were limited with the information available in existing patient records. Information on age at diagnosis (years); sex; race (white, African American, Asian, Native American/Alaskan, Hawaiian/Pacific Islander, other); ethnicity (Hispanic, non-Hispanic); alcohol use (never, ever) and cigarette, pipe, or cigar smoking status (never, former, current); tumor stage (I-IV); and tumor differentiation (poor, moderate, well) was available electronically from the Roswell Park Cancer Institute Hospital Information System. Information on missing teeth was obtained from radiographs.

To compare HPV-positive cases and HPV-negative controls for similarity, the Mann-Whitney test for continuous variables and Fisher exact test for categorical variables were used. We have performed receiver operating characteristic curve analysis to identify a cut point for ABL for the definition of periodontitis. The independent effect of periodontitis on tumor HPV status was estimated from multiple logistic regression analysis after adjusting the effects of age at diagnosis, sex, race/ethnicity, smoking status, alcohol use, and number of missing teeth. Odds ratios and their 95% confidence intervals were calculated. A 2-sided $\alpha < .05$ was set to indicate statistical significance. SPSS software, version 15.0 (SPSS Inc, Chicago, Illinois) was used for data analyses.

RESULTS

Thirty new cases of primary base of tongue squamous cell carcinoma were diagnosed between June 15, 1999, and January 10, 2005, for whom we had both periodontal and pathology data available. The prevalence of tumors positive for HPV-16 DNA was 21 of 30 (70%). None of the tumor samples were positive for HPV-18 DNA. According to the receiver operating characteristic curve analysis, periodontitis was defined as ABL of 2.74 mm or greater. Compared with participants with HPV-negative tumors, those with HPV-positive tumors had significantly higher mean ABL (3.90 mm vs 2.85 mm, $P = .01$) and a greater prevalence of periodontitis (86% vs 22%, $P = .002$). Remaining variables—age, sex, race/ethnicity, smoking, alcohol use, tumor stage, and tumor differentiation—were not significantly different between cases and controls (**Table 1**).

Stratification of the periodontitis-HPV association by smoking status revealed that the history of periodontitis was associated with HPV-positive tumor status in smokers and nonsmokers. On the other hand, the effect of smoking on tumor HPV status depended on periodontitis history. All (6 of 6) of the never-smokers had HPV-positive tumors and 83% (5 of 6) of them had a history of periodontitis. Among smokers, 87% (13 of 15) of those with HPV-positive tumors had a history of periodontitis, and 78% (7 of 9) of those with HPV-negative tumors had no history of periodontitis (**Table 2**). Stratification of the periodontitis-HPV association by alcohol use yielded similar results: Among patients with HPV-positive tumors, 77% (10 of 13) of the nondrinkers and all of the drinkers (8 of 8) had periodontitis. Among patients with HPV-negative tumors, 75% (3 of 4) of the nondrinkers and 80% (4 of 5) of the drinkers had no history of periodontitis (**Table 3**).

Every millimeter of ABL was associated with an approximately 3-fold (odds ratio, 2.86; 95% confidence interval, 1.03-7.98) increased risk of HPV-positive tumor status. Number of missing teeth was not significantly associated with tumor HPV status (0.94; 0.86-1.12). After adjustment for age at diagnosis, sex, race/ethnicity, alcohol use, smoking status, and number of missing teeth, each millimeter of ABL was associated with an approximately 4-fold (3.96; 1.18-13.36) increased risk of HPV-positive tumor status (**Table 4**). The model explained 76% of the variation in tumor HPV status.

COMMENT

Chronic periodontitis may play a significant role in the natural history of HPV infection in patients with base of tongue cancer. Despite a small sample size, the association was statistically significant. These results encourage larger epidemiologic studies to confirm this association and basic science studies to test the roles of chronic inflammation and bacterial coinfections in the natural history of oral HPV infection.

Infection of hosts by multiple microorganisms is the norm rather than the exception, yet much research has focused on single pathogens. Studies suggest that viruses and bacteria, mostly studied in isolation, may in fact cooperate synergistically and should be considered as a pathogenic consortium.[3,10,11,17] The prevalence and severity of both periodontitis and HPV infection are significantly higher in patients infected with HIV and herpesviruses.[18,19] This provides additional indirect evidence for periodontitis-HPV association. It was shown that herpesvirus infections occurred more frequently in

Table 1. Description of the Study Population by Tumor HPV Status

	HPV-Negative Tumors (n=9)	HPV-Positive Tumors (n=21)	*P* Value[a]
Age at diagnosis, y, mean (SD)	59.8 (11.6)	57.1 (9.4)	.56
Sex, No. (%)			
Female	3 (33)	1 (5)	.07
Male	6 (67)	20 (95)	
Race, No. (%)[b]			
Non-Hispanic white	8 (89)	18 (86)	.54
Hispanic white	1 (11)	1 (5)	
Non-Hispanic African American	0	2 (10)	
Smoking status, No. (%)			
Never	0	6 (29)	.14
Ever	9 (100)	15 (71)	
Alcohol use, No. (%)			
Never	4 (44)	13 (62)	.44
Ever	5 (56)	8 (38)	
TNM stage, No. (%)			
I-II	0	2 (10)	>.99
III-IV	7 (78)	19 (90)	
Unknown	2 (22)	0	
Tumor differentiation, No. (%)			
Poorly	5 (56)	9 (43)	.68
Moderate to well	3 (33)	11 (52)	
Unknown	1 (11)	1 (5)	
Periodontitis, No. (%)[c]			
No	7 (78)	3 (14)	.002
Yes	2 (22)	18 (86)	
ABL, mm, mean (SD)	2.85 (0.97)	3.90 (1.17)	.01
Missing teeth, mean (SD)	9.22 (6.26)	6.81 (6.89)	.17

Abbreviations: ABL, alveolar bone loss; HPV, human papillomavirus.
[a] *P* values were derived from Mann-Whitney test for continuous variables and the Fisher exact test for categorical variables.
[b] Percentage may not total 100 because of rounding.
[c] Periodontitis is defined as ABL ≥2.74 mm.

Table 2. Periodontitis-HPV-Smoking Status Cross-tabulation

	HPV-Negative Tumors (n=9)	HPV-Positive Tumors (n=21)
Never-smoker, No. (%)		
No periodontitis[a]	0	1 (17)
Periodontitis	0	5 (83)
Ever-smoker, No. (%)		
No periodontitis	7 (78)	2 (13)
Periodontitis	2 (22)	13 (87)

Abbreviation: HPV, human papillomavirus.
[a] Periodontitis is defined as alveolar bone loss ≥2.74 mm.

Table 3. Periodontitis-HPV-Alcohol Use Cross-tabulation

	HPV-Negative Tumors (n=9)	HPV-Positive Tumors (n=21)
No alcohol use, No. (%)		
No periodontitis[a]	3 (75)	3 (23)
Periodontitis	1 (25)	10 (77)
Alcohol use, No. (%)		
No periodontitis	4 (80)	0
Periodontitis	1 (20)	8 (100)

Abbreviation: HPV, human papillomavirus.
[a] Periodontitis is defined as alveolar bone loss ≥2.74 mm.

periodontitis sites and that herpesvirus-associated periodontal pockets harbored higher levels of periodontal bacteria. In addition, periodontal disease severity was significantly associated with salivary viral load, and periodontal treatment reduced the viral load to undetectable levels.[20] It was suggested that periodontitis sites are a source of salivary herpesviruses and that periodontal treatment may help control viral replication, transmission, and associated diseases.[11] Inflammatory diseases such as periodontitis also enhance HIV viral load because increased inflammation attracts HIV-infected lymphocytes and monocytes.[21] In vitro experiments show that HIV replication is finely regulated by various inflammatory cytokines acting in autocrine and paracrine manner. Interleukins 1 and 6 and tumor necrosis factor α have been demonstrated to induce HIV expression and replication in latently infected, resting $CD4^+$ T cells.[20] Increased inflammatory infiltrate carries HIV- and herpesvirus-infected cells to the periodontitis sites, where cellular breakdown sheds viruses into the oral cavity.[21] Saliva provides a means of interaction between shed viruses, bacteria, inflammatory mediators, and other carcinogens such as tobacco and alcohol metabolites.[22] For example, bacterial production of acetaldehyde from ethanol is increased in patients with poor oral health.[23] Saliva also provides a means of transport from one surface to another, promoting disease at distant sites.[24] This may be related to the field cancerization in the head and neck region because more than 75% of oral cancers are found in saliva drainage areas.

Table 4. Odds Ratios for Tumor Human Papillomavirus Status Associated With Periodontal Variables[a]

	Crude Odds Ratio (95% CI)	*P* Value	Adjusted Odds Ratio (95% CI)[b]	*P* Value
ABL, per mm	2.86 (1.03-7.98)	.04	3.96 (1.18-13.36)	.03
Missing teeth, per tooth	0.94 (0.86-1.02)	.14	0.95 (0.74-1.21)	.67

Abbreviations: ABL, alveolar bone loss; CI, confidence interval.
[a] Periodontitis is defined as ABL of ≥2.74 mm.
[b] Logistic regression model included age at diagnosis, sex, race/ethnicity, alcohol use, smoking status, ABL, and number of missing teeth.

The association between smoking and HPV prevalence in the literature is inconsistent. Although recent studies have shown that never-smokers tend to have HPV-positive tumors, most patients with HPV-positive tumors are still smokers.[25] Therefore, defining a relationship between the virus, smoking, and other host and environmental influences may be essential to understand HPV-associated carcinogenesis. In our study, smoking alone was not a good predictor of tumor HPV status. All never-smokers had HPV-positive tumors, confirming the results of previous studies. However, more than half of the smokers also had HPV-positive tumors. Stratification by periodontitis history revealed that most of the never-smokers with HPV-positive tumors had periodontitis. Among smokers, those who had periodontitis were more likely to have HPV-positive tumors and those without periodontitis were more likely to have HPV-negative tumors. Periodontitis-HPV-alcohol use cross-tabulation yielded similar results, suggesting that the associations between HPV, smoking, and alcohol are modified by periodontitis. Therefore, stratification by local factors, such as chronic inflammation or bacterial infections, may provide valuable insights into the association of HPV with tobacco and alcohol use.

It is important to understand the difference between gingivitis and periodontitis, although both are commonly called "gum disease." Gingivitis is not a destructive process; it is reversible and affects everybody with poor oral hygiene. On the other hand, only a small subset of the population with poor oral hygiene develops destructive periodontitis, leading to epithelial migration and irreversible tissue loss.[10,11] Factors that initiate periodontitis are not well understood. Recent evidence supports a significant role of viruses in the initiation and progression of periodontitis.[10,11,18] Smoking reduces gingivitis, but it is a strong risk factor for periodontitis.[10] A link between poor oral hygiene and HNCs has been suggested for a long time without a definitive answer.[26] It is possible that among patients with poor oral hygiene, only those who develop periodontitis are at high risk for HNC. Clinical studies that include both patients with gingivitis and patients with periodontitis will allow testing of this hypothesis.

A strength of this study is that we had objective and quantitative documentation of periodontitis history before cancer diagnosis. The radiographs were taken at admission. However, detectable ABL on radiographs reflects preexisting longstanding periodontitis, often representing decades of history in older populations.[10] On the other hand, we cannot conclude from this study that periodontitis preceded HPV infection. These 2 infections may have occurred concurrently, or HPV infection may have preceded periodontitis. A prospective clinical study or nested case-control study is needed to assess the temporal relationship of these 2 infections.

A disadvantage of secondary data analyses is that they depend on availability of data in adequate detail from preexisting medical records. In this study, limited information was available on covariates. For example, data on smokeless tobacco, diet, and sexual habits was not available. However, basic information on age, sex, race/ethnicity, alcohol and tobacco use, histologic confirmation of tumor diagnoses, sensitive assays to detect tumor HPV DNA, and quantitative and objective measures of periodontitis history was available.

Our study population was hospital based and consisted of patients with cancer, differing from the general population in a number of ways. Therefore, we cannot generalize our results and make inferences about the natural history of HPV infection in healthy populations. On the other hand, using a high-risk homogeneous population from the same source increases the efficiency of the study and reduces selection bias. We hope that the results of this study will stimulate other researchers who have access to larger populations, including cancer-free participants.

Years of research on specific microbial etiologies has not translated into effective treatment strategies for cancer. In most cases, the mere presence of a microorganism is not sufficient to cause cancer. In addition, biofilm formation by bacteria and the intracellular nature of viruses protect them from host defense and chemotherapy. Our approach is to clinically identify those infected individuals who are at a high risk of developing HNC. If periodontitis-HPV synergy is real, it will offer insights into the natural history of HPV infection and will translate immediately into clinical practices for improved prevention and treatment strategies. Currently, there is no treatment for HPV infection. On the other hand, treatment of periodontitis is safe and may reduce the acquisition, transmission, and persistence of HPV. The results of this study provide insights for larger studies, including clinical variables and biomarkers of periodontal disease.

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Author Contributions: Drs Tezal, Sullivan Nasca, and Hyland had full access to all of the data in the study and take responsibility for the integrity of the data and the

accuracy of the data analysis. *Study concept and design:* Tezal and Loree. *Acquisition of data:* Tezal, Sullivan Nasca, Stoler, Smaldino, and Rigual. *Analysis and interpretation of data:* Tezal, Stoler, Melendy, Hyland, Smaldino, and Loree. *Drafting of the manuscript:* Tezal and Smaldino. *Critical revision of the manuscript for important intellectual content:* Sullivan Nasca, Stoler, Melendy, Hyland, Rigual, and Loree. *Statistical analysis:* Hyland. *Obtained funding:* Tezal. *Administrative, technical, and material support:* Tezal, Sullivan Nasca, Stoler, Smaldino, Rigual, and Loree. *Study supervision:* Melendy and Loree.

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