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Immunohistochemical detection of p16^{INK4a} in dysplastic lesions of the oral cavity

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Significant intra- and interobserver variability exists in diagnosing and grading oral epithelial dysplasia. Mutations in the tumor-suppressor gene *p16* are common in oral cavity dysplastic lesions, but whether immunohistochemical detection of the gene product p16^{INK4a} (p16) can be used as a reliable biomarker for dysplasia is unclear. In total, 119 biopsy specimens representing various oral cavity sites and degrees of dysplasia were retrieved from the pathology files of Emory University Hospital. Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and with a monoclonal antibody to p16 (LabVision Corporation, Clone JC2). A blinded review of the H&E slides and the pattern and degree of p16 expression was independently performed by two pathologists. A consensus was obtained when diagnoses differed. Morphologic diagnoses were then compared to p16 immunohistochemical expression. Overall, 61/119 (51%) cases showed no p16 immunoreactivity, including 12/33 (36%) cases of no dysplasia, 11/28 (39%) cases of mild dysplasia, and 38/58 (66%) cases of moderate/severe dysplasia. The remaining cases showed p16 expression limited to the basal and suprabasal nuclei and generally confined to the lower one-third of the epithelium. A logistic regression model showed a trend toward absent p16 expression with increasing severity of dysplasia ($P = 0.006$). Decreased expression of p16 in dysplastic lesions, as found in this study, may reflect the biologic events involving loss of *p16* gene function in the pathogenesis of oral cancer. Our findings suggest that p16 immunohistochemistry is not helpful in differentiating dysplastic from nondysplastic mucosa in oral cavity biopsies, and thus is not a reliable biomarker for use in routine clinical practice.
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Worldwide there were an estimated 274 000 new cases of oral cavity cancer in 2002, with almost two-thirds occurring in men,[1] and the overwhelming majority are squamous cell carcinomas. For many years it has been known that invasive squamous cell carcinoma is preceded by a progressive accumulation of genetic mutations within the epithelial cells.[2] Premalignant changes are often clinically apparent as leukoplakia or erythroplakia, and histologically are identified as dysplasia. In a review by Barnes, the risk for developing invasive squamous cell carcinoma in laryngeal mucosa for patients with mild, moderate, and severe dysplasia was 5.7, 22.5, and 28.4%, respectively.[3] Thus, there is a five-fold increased risk of developing invasive carcinoma for severe compared to mild dysplasia, which highlights the need for accurate histologic grading of clinically suspicious lesions.

Although multiple different grading systems have been proposed, the generally preferred scheme for dysplastic lesions of the oral cavity includes mild, moderate, and severe dysplasia. Grading of oral dysplasia is based on architectural, cytomorphologic, and maturation abnormalities including irregular epithelial stratification, drop-shaped rete processes, loss of polarity of the basal cells, single cell keratinization, increased nuclear/cytoplasmic ratio, increased number of mitotic figures, cellular and nuclear pleomorphism, enlarged nucleoli, and loss of cellular cohesion.[4] While these histologic features are widely known, multiple studies have shown that intra- and interobserver agreement in the diagnosis and grading of oral dysplasia is generally poor.[5–9]

Tumor-suppressor gene inactivation has been associated with oral dysplasia and squamous cell carcinoma, most commonly involving loci on chromosomes 3p, 9p, and 17p, although many others have been described.[2,10–23] The function of the tumor-suppressor genes located on chromosome 3p

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are currently poorly defined, whereas the functions of *p16* (also known as *CDKN2A*, located on 9p21) and *p53* (located on 17p13) are well established. Inactivation of these genes causes altered expression of their respective gene products $p16^{INK4a}$ (p16) and p53, which suggests that they may be useful biomarkers for oral cavity dysplasia and carcinoma.[20,21,24–27]

Immunohistochemical detection of p16 has been shown to be a reliable marker for squamous dysplasia in the uterine cervix, where the protein is frequently overexpressed as a result of infection with oncogenic strains of human papillomavirus (HPV).[28–32] More recently, a study showed immunohistochemical overexpression of p16 in dysplastic lesions of the oral cavity, which suggests that it may be a useful ancillary test for diagnosing dysplasia in this location.[33] However, several other studies have shown decreased p16 expression in oral premalignant and malignant lesions by immunohistochemistry, which has been attributed to gene inactivating mechanisms such as homozygous deletion, point mutation, and promoter hypermethylation.[20,21,27,34] The purpose of the current study was to further investigate p16 immunohistochemistry as a potential biomarker for dysplasia using a large series of oral cavity biopsies with and without dysplasia with the goal of determining its usefulness for routine clinical practice.

Materials and methods

Case Selection

This study was approved by the Emory University Institutional Review Board. In total, 119 biopsy specimens accessioned between April 2002 and July 2005 representing various oral cavity sites and degrees of dysplasia (Table 1) were retrieved from the pathology files of Emory University Hospital. Only well-characterized cases of normal and dysplastic mucosa were selected so that the patterns of p16 staining could be reliably compared to degree of dysplasia. Poorly oriented biopsies and biopsies exhibiting ulceration were excluded.

p16 Immunohistochemistry

For each case, a 4-μm-thick section of formalin-fixed, paraffin-embedded tissue was stained with hematoxylin and eosin (H&E), and an adjacent section was immunostained for the presence of p16 (mouse monoclonal antibody, clone 16P04, 1:40 dilution, LabVision, Fremont, CA, USA) using heat-induced antigen retrieval, horseradish peroxidase (HRP)-labeled polymer conjugated with secondary antibodies (Dako Envision System, Carpinteria, CA, USA), and the Dako Autostainer (Dako, Carpinteria, CA, USA). A section of vaginal squamous cell carcinoma was used as the positive control. Negative controls had primary antibody replaced by buffer.

Tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed in citrate buffer (pH 6) using an electric pressure cooker for 5 min at 120°C, with cooling for 10 min before immunostaining. Tissues were then exposed to 3% hydrogen peroxide for 5 min to block endogenous peroxidase, followed by appropriately characterized and diluted primary antibody for 30 min, HRP-labeled polymer for 30 min, diaminobenzidine as chromogen for 5 min, and Dako automation hematoxylin as counterstain for 15 min. These incubations were performed at room temperature. Between incubations, sections were washed with Tris-buffered saline (TBS) buffer. Coverslipping was performed using the Tissue Tek SCA automatic coverslipper (Sakura Finetek USA Inc., Torrance, CA, USA).

Evaluation of Cases for Dysplasia and Immunohistochemical Staining

Each recut H&E slide was independently examined by two pathologists (SDB and SL) and graded as no, mild, moderate, or severe dysplasia without knowledge of the original sign-out diagnosis. The percentage of cases showing exact agreement between the two observers was calculated, as were levels of disagreement. A consensus grade was determined by joint review for cases in which individual diagnoses differed. However, consensus was not attempted when one pathologist diagnosed moderate dysplasia and the other diagnosed severe dysplasia because there is relatively less clinical importance in distinguishing these two grades since they are often managed similarly.

p16-stained slides were reviewed independently by the same two pathologists without knowledge

Table 1 Summary of study cases

Original sign-out diagnosis	*Oral cavity sites (no. of cases)*							
	Tongue	*Floor of mouth*	*Buccal mucosa*	*Palate*	*Gingiva*	*Lip*	*Unspecified*	*Total*
No dysplasia	8	3	6	5	6	1	1	30
Mild dysplasia	15	6	6	2	0	0	1	30
Moderate dysplasia	21	4	2	2	0	1	0	30
Severe dysplasia	16	6	1	3	0	3	0	29

of the H&E grade. Positive cases were defined as having five or more squamous epithelial cells with staining of the nucleus, cytoplasm, or both. Positive cases were divided into two semiquantitative groups: basal layer staining, in which staining was confined to the basal cells with no staining or very rare staining of more superficial cells; and basal and suprabasal layer staining, defined as positivity of cells in both the basal and suprabasal layers. Consensus was obtained when individual interpretations differed.

To verify the technical validity of our results, p16 immunohistochemical staining was repeated for 15 of the 119 cases using the identical technique as described above. Cases were randomly chosen from those diagnosed as moderate/severe dysplasia. Staining patterns for these cases were interpreted by one of us (SL) without knowledge of the previous staining pattern. The same criteria were used for interpretation as described above.

Statistical Analysis

For statistical analyses, p16-negative cases were compared to p16-positive cases for each degree of dysplasia. Positive cases were grouped together because the difference between basal layer staining and basal plus suprabasal layer staining, while potentially informative histologically, is undefined in terms of p16 inactivation. A χ^2 test of independence was used to test the null hypothesis that immunohistochemical expression of p16 is unrelated to degree of dysplasia. Trends in the data were assessed using a proportional odds ordinal logistic regression model. A P-value of <0.05 was used to indicate statistical significance.

Results

Dysplasia Grading

Consensus histologic grading for the 119 cases included in this study showed there to be 33 cases with no dysplasia, 28 cases with mild dysplasia, and 58 cases with moderate/severe dysplasia (Table 2, Figure 1). There was exact agreement in grade of dysplasia between the two observers for 69 of 119 cases (58%) including 28 cases diagnosed as no dysplasia, 12 cases of mild dysplasia, nine cases of moderate dysplasia, and 20 cases of severe dysplasia. There was agreement within one histologic grade for 117 of 119 cases (98%). Overall, this level

Table 2 p16 immunohistochemistry results

Consensus H&E grade	*p16 expression*			
	Negative	*Basal layer staining*	*Basal and suprabasal layer staining*	*Total number of cases*
No dysplasia	12 (36%)	18 (55%)	3 (9%)	33 (100%)
Mild dysplasia	11 (39%)	12 (43%)	5 (18%)	28 (100%)
Moderate/severe dysplasia	38 (66%)	15 (26%)	5 (9%)	58 (100%)

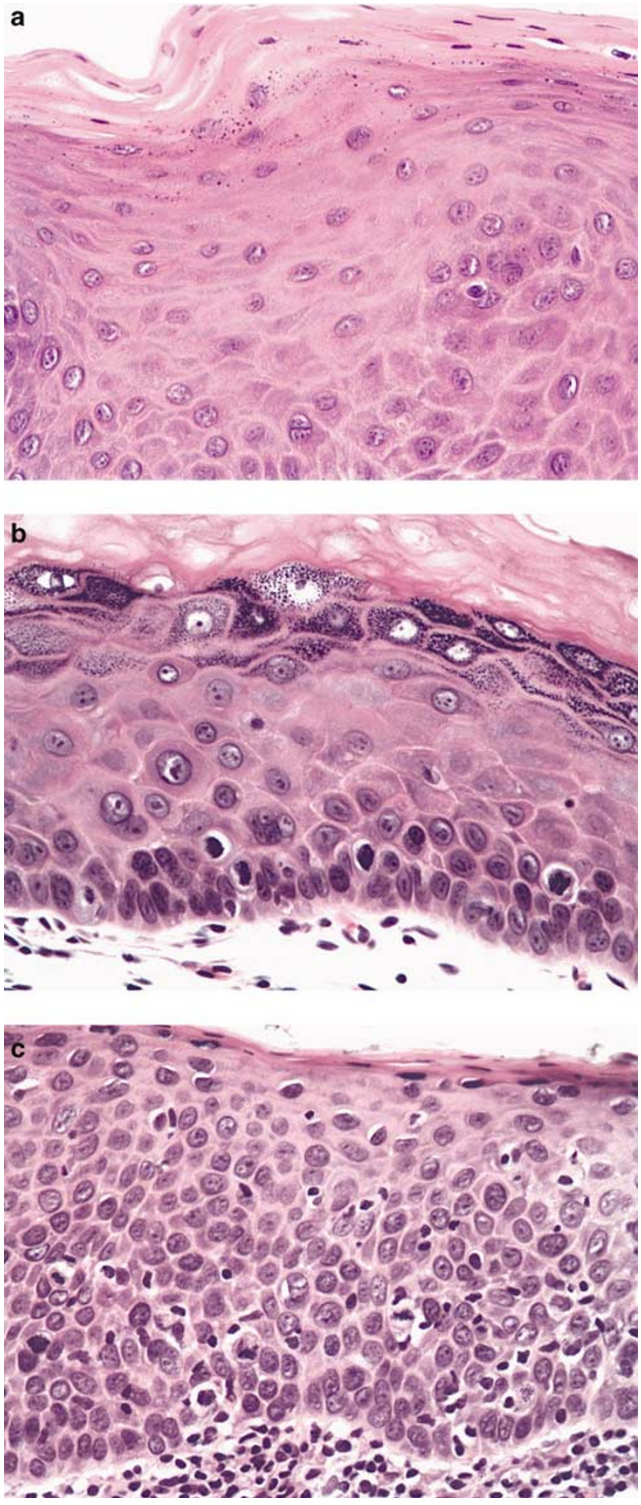

Figure 1 Representative H&E consensus histologic grades of oral cavity biopsies: (**a**) no dysplasia; (**b**) mild dysplasia; (**c**) moderate/severe dysplasia.

of agreement is higher than that typically reported in the literature, which likely reflects the fact that only cases of well-characterized dysplasia were included during case selection.

p16 Immunohistochemistry

Overall, 61 of 119 cases (51%) were negative for p16 immunoreactivity, including the majority of cases diagnosed as moderate/severe dysplasia (Table 2). In the remaining cases, p16 expression was limited to the basal and suprabasal cell layers and generally was confined to the lower one-third of the epithelium. Representative examples of the immunohistochemical staining patterns are shown in Figure 2. The positive control was strongly and diffusely positive (Figure 2). χ^2 analysis showed p16 expression to be dependent on severity of dysplasia ($\chi^2 = 9.26$, df $= 2$, $P = < 0.01$) with a significant trend toward absent p16 expression with increasing severity of dysplasia (logistic regression model, $\beta = -0.62$, $P = 0.006$). For the 15 cases in which p16 immunohistochemistry was repeated, each showed a staining pattern that was similar to the original run.

Discussion

Oncogenesis in the oral cavity is widely believed to result from cumulative genetic alterations that cause a step-wise transformation of the mucosa from normal to dysplastic to invasive carcinoma.[2] Attempts have been made to correlate the specific genetic changes with histopathological progression, but this has remained a challenge since numerous genes are involved.[2] In a review, Patel *et al*[17] summarized the chromosomal abnormalities that are commonly found in oral cancer, including losses of 3p, 4q, 5q21–22, 8p21–23, 9p21–22, 11q13, 11q23, 13q, 14q, 17p, 18q, and 22q. Of these, loss of chromosomal 9p is frequently reported to be the most common genetic abnormality in oral dysplasia and carcinoma.[2,11,15] Various studies have shown

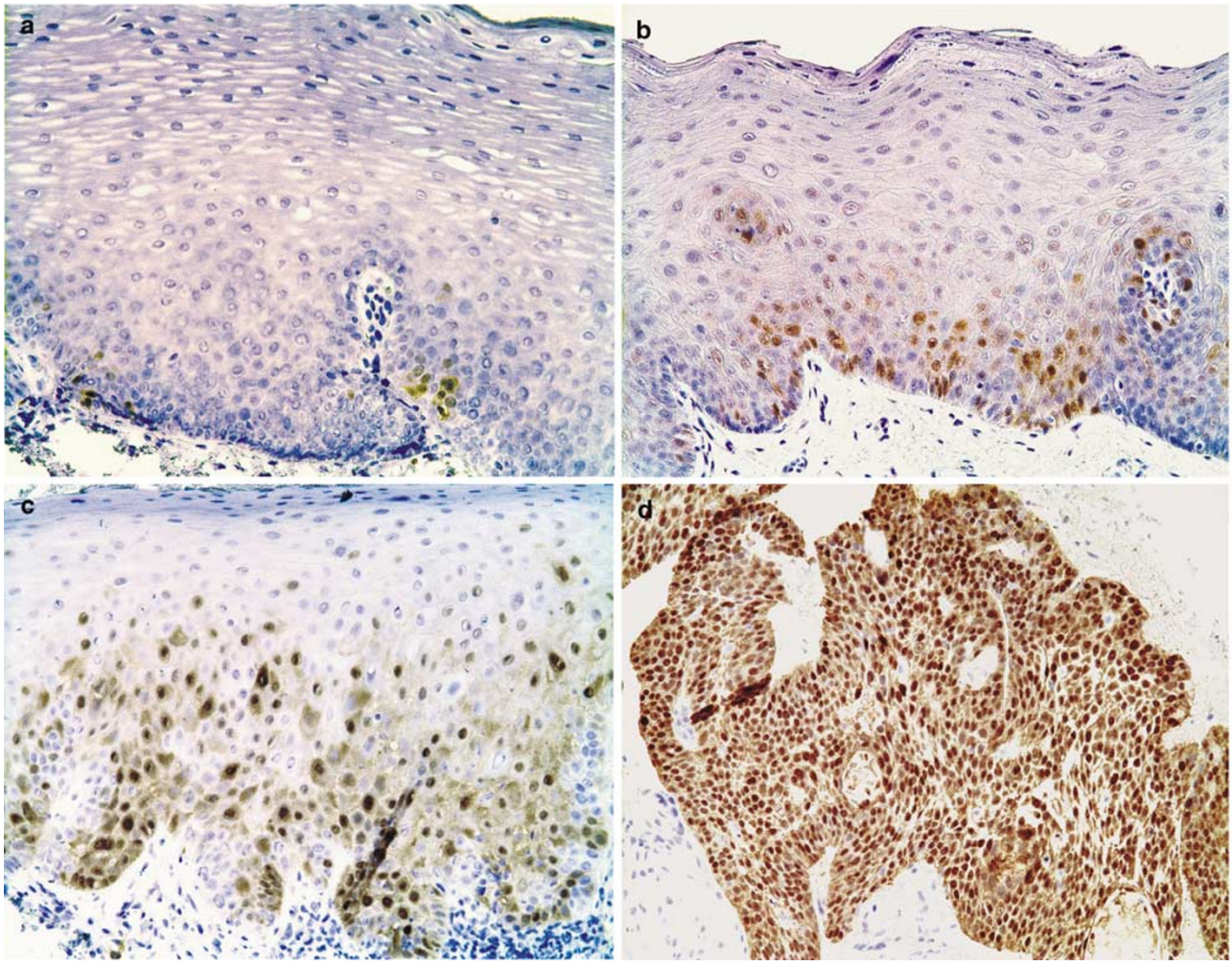

Figure 2 Representative immunohistochemical staining patterns of oral cavity biopsies using a p16 monoclonal antibody: (**a**) basal layer staining; (**b**) basal and suprabasal layer staining. (**c**) The most strongly positive case showed focal areas with p16 staining of the mid to upper cell layers; however, adjacent areas on the same slide showed no p16 expression. (**d**) The p16 positive control is strongly and diffusely positive (vaginal squamous cell carcinoma).

loss of heterozygosity on 9p21 in 20% of benign squamous hyperplasias,[2] 28–71% of dysplastic lesions,[2,11,14,22] and 19–73% of invasive squamous cell carcinomas.[2,11,13–15,25,35] In addition, because invasive carcinomas often do not exhibit increased loss of heterozygosity compared to preinvasive lesions, loss of 9p is considered an early event in tumor progression.[2,14]

The tumor-suppressor gene *p16* is localized on 9p21, and its inactivation is considered to be a significant event in development of many tumor types including oral carcinoma,[36] although one study showed that additional tumor-suppressor genes may exist at this locus.[35] The protein gene product p16 normally binds to cyclin-dependent kinases (cdk) 4 and 6, inhibiting their association with cyclin D1. The inhibition of the cdk 4/6-cyclin D1 complex prevents phosphorylation of the retinoblastoma protein (pRb) leading to inhibition of cell cycle progression through G_1- to S-phase.[10] While inactivation of pRb is variable in oral squamous cell carcinoma, inactivation of *p16* is common and usually occurs via homozygous deletion, point mutation, or promoter hypermethylation.[13,16,20,21,27,34,37,38]

The frequent occurrence of *p16* inactivation during early carcinogenesis has led to its investigation as a surrogate marker for dysplasia. The diagnosis of oral dysplasia is currently dependent on interpretation of H&E-stained slides, but this is known to be subjective with considerable intra- and interobserver variability.[5–9] Immunohistochemical evaluation of oral premalignant and malignant lesions for p16 expression using an anti-p16 antibody has given variable results with some studies showing decreased expression[20,21,27,34] and others showing overexpression.[33,39] The two studies showing overexpression and one study showing decreased expression[27] each utilized a polyclonal antibody (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), while the remaining studies used various monoclonal antibodies. In our experience, the C-20 polyclonal antibody produces nonspecific staining including prominent muscle staining and positivity of both dysplastic and nondysplastic oral mucosa (unpublished data). Prior studies have shown excellent concordance between *p16* gene inactivation as determined by molecular methods and p16 protein expression detected using immunohistochemistry.[20,21,27,34] Thus, the frequent loss of the *p16* gene in dysplastic mucosa would be expected to result in a decrease in p16 expression compared to normal mucosa.

In the current study, we used a monoclonal antibody to investigate the immunohistochemical expression of p16 in oral mucosa biopsies showing varying degrees of dysplasia. Our data show a significant trend toward absent expression of p16 with increasing severity of dysplasia. However, loss of p16 expression was not limited to dysplastic lesions, since a significant percentage of nondysplastic mucosa was also negative. Furthermore, when present, staining was confined to the basal and suprabasal cell layers in both normal and markedly dysplastic mucosa with no cases showing full-thickness positivity. These findings indicate heterogeneous expression of p16 within morphologically homogeneous tissue. In addition, our data shows that p16 expression cannot reliably differentiate normal from dysplastic mucosa.

Our finding of variable p16 expression in oral cavity biopsies is in contrast to the uterine cervix where squamous dysplastic lesions frequently overexpress p16 as a result of infection with high-risk HPV types. This has resulted in the use of p16 immunohistochemistry as a biomarker of cervical dysplasia.[28–32] In the cervix, p16 overexpression is thought to be the result of inactivation of pRb by the HPV E7 oncoprotein. The interaction of E7 with pRb results in release of the transcription factor E2F from the active pRb-E2F complex. As pRb-E2F normally inhibits transcription of the *p16* gene, expression of HPV E7 results in excessive and deregulated transcription and translation of *p16*.[40] Thus, the mechanism for p16 overexpression in HPV-related cervical dysplastic lesions is thought to be different from the mechanism of *p16* inactivation, which is more common in oral cavity dysplasia.

Using monoclonal p16 immunohistochemistry in oral malignancies, Fregonesi *et al*[41] showed a strong association between overexpression of p16 and infection with high-risk HPV types. Their study demonstrated diffuse p16 positivity in 69% of HPV 16/18 cases compared to 4% of HPV-negative cases and 0% of HPV 6/11 cases, although focal or sporadic staining was seen in many of the HPV-negative and HPV 6/11 cases.[41] In addition, a study by Saito *et al*[26] showed overexpression of p16 in 45% of oral verrucous carcinomas, which are etiologically often associated with HPV infection, compared to only 11% of cases of usual squamous cell carcinoma. These findings have led to the suggestion that overexpression of p16 may serve as a biomarker for HPV-induced oral dysplasia or carcinoma. However, the incidence of HPV infection in oral mucosa is variable,[42–45] which implies that immunohistochemistry is likely to be unreliable without knowledge of HPV status. As HPV status was not evaluated in the current study, it is unclear whether our results are associated with a low prevalence of high-risk HPV infection in the population studied.

Decreased immunohistochemical expression of p16 in dysplastic lesions of the oral cavity, as found in this study, may be due to *p16* inactivation, although this was not tested. Additional studies investigating *p16* gene inactivation and high-risk HPV status concurrently with immunohistochemical detection of p16 would be helpful in clarifying the patterns of p16 expression observed in dysplastic mucosae. Regardless of the mechanism involved, our findings suggest that p16 immunohistochemistry is not helpful in differentiating dysplastic from

nondysplastic mucosa in oral cavity biopsies, and thus is not a reliable biomarker for use in routine clinical practice.

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Duality of interest

None declared.

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