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Atypical Protein Kinase C λ/ι Expression Is Associated with Malignancy of Oral Squamous Cell Carcinoma

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Abstract. *Background/Aim: Atypical protein kinase C λ/ι (aPKCλ/ι) is a cell polarity-regulator localized in the tight junction and apical membrane in epithelial cells. Previous studies suggested that aPKCλ/ι overexpression and abnormal localization were involved in tumor progression in several cancers. We investigated the relationship between aPKCλ/ι and oral squamous cell carcinoma (OSCC). Materials and Methods: The correlation between the aPKCλ/ι expression and the clinicopathological parameters in 76 OSCC cases was examined using immunohistochemical analyses. Results: aPKCλ/ι overexpression was observed in 36.8% of cases. aPKCλ/ι expression was more intense in poorly differentiated OSCC and younger patients (<60 years of age). Although expression of aPKCλ/ι was not significantly associated with clinical parameters, the correlation was found between aPKCλ/ι localization and progression-free survival. Conclusion: This is the first study to assess the association of aPKCλ/ι expression in OSCC with clinical results. Expression and localization of aPKCλ/ι may be involved in the degree of malignancy in OSCC.*

Head and neck cancer is the sixth most common cancer worldwide. Annually, 280,000 people develop head and neck cancer and 160,000 die of the disease (1). Oral cancer is the most common head and neck cancer. In Japan, oral cancer is the 11th most common cancer and accounts for only 1-2% of all cancers (2). However, the number of cases has been increasing in recent decades, with 8,000 people developing the disease and more than 7,000 patients dying annually (2). Ninety percent of oral cancers are histologically squamous cell carcinoma, followed by adenocarcinoma and sarcoma, accounting for the remaining 10%. Oral squamous cell carcinoma (OSCC) exhibits a high rate of lymph node metastasis. Locoregional recurrence is the major prognostic factor for mortality. The overall 5-year survival rate for OSCC patients remains approximately 50%, which has not significantly improved over several decades. Patients with OSCC are clinically treated with surgery as a standard therapy and/or radiation therapy and chemotherapy as multimodality therapy. However, postoperative dysfunction such as dysphagia, dysphemia, or cosmetic disturbance sometimes occurs, which can reduce the quality of life, especially for advanced cases. Therefore, early diagnosis and conservative therapy are important in the treatment of patients with OSCC to avoid such dysfunction. However, some cases, even those diagnosed at an early stage and are appropriately treated, have shown rapid metastasis and poor prognosis. Currently, there has been no reliable biomarker of OSCC to identify such cases at the stage of the first presentation. We focused our attention on atypical protein kinase C λ/ι (aPKCλ/ι), which has a role in cell polarity, as a clue to the cause of rapid metastasis.

aPKCλ/ι is a multifunctional protein in normal epithelial cells, with roles in cell survival, cell growth, and control of signal transduction (3-5). In addition, aPKCλ/ι protein localizes at the cell-cell junction and apical domain of

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several epithelial cell types. It also plays an important role in the establishment of cell polarity (3, 6). Cancer cells show aberration of cellular morphology and disturbed cell polarity. In fact, overexpression of aPKCλ/ι has been reported in several types of cancer, including lung, breast, stomach, ovary, colon, and prostate cancer (7-15). Despite its well-known involvement in some tumor types, the expression and role of aPKCλ/ι in OSCC remain to be clarified.

In this study, we investigated the correlation between the aPKCλ/ι expression pattern and the clinicopathological factors in 76 OSCC cases and considered its potential value as a biomarker for early detection and/or degree of malignancy in OSCC.

Materials and Methods

Patients and clinicopathological data. The protocol of this research was approved by the Institutional Ethical Committee of the Yokohama City University in accordance with the principles of the Declaration of Helsinki. This study included 76 patients with OSCC who underwent treatment between April 2007 and March 2010 at the Department of Oral and Maxillofacial Surgery, Yokohama City University Hospital, Yokohama, Japan. Written informed consent was obtained from all patients enrolled in this study for the use of their tissue samples. All the tissue samples were selected from the patients who did not have preoperative therapy. Clinicopathological parameters (age, sex, smoking history, clinical stage, lymph node metastasis, and pathological type) were obtained from archival records. Formalin-fixed, paraffin-embedded sections were routinely stained with hematoxylin and eosin. Pathological tumor-node-metastasis (pTNM) stages were determined according to the Union for International Cancer Control (UICC) 7th edition. Pathological types were determined by two experienced pathologists (Y.N. and Y.I.), according to the World Health Organization.

Immunohistochemistry. For immunohistochemistry, 4-μm-thick formalin-fixed, paraffin-embedded sections were prepared according to the methods previously described (7). Briefly, the sections were deparaffinized, rehydrated in ethanol, and autoclaved in 10 mM citrate buffer (pH 6.0) for antigen retrieval. The sections were then immersed in 0.3% hydrogen peroxide to quench the intrinsic peroxidase activity. After incubation with 10% normal rabbit serum, the sections were incubated with the anti-aPKC ι antibody (diluted 1:500; BD Transduction Laboratories, San Diego, CA) at 4˚C overnight. The labeled antigens were visualized with the HistoFine (Nichirei Pharmaceutical, Tokyo, Japan) and DAB plus kits (DAKO Cytomation, Kyoto, Japan). Finally, the sections were counterstained with hematoxylin and microscopically examined. Normal oral squamous epithelial tissue from a healthy part of the specimens obtained from the same patient was simultaneously examined as a control.

Scoring the expression levels in immunohistochemistry. Two investigators (J.B. and Y.N.) independently assessed the stained sections. The positive signals for aPKCλ/ι were semi-quantitatively scored according to the following criteria: 0, no staining; 1, weak to normal staining (equivalent with normal oral squamous epithelium); 2, moderate staining; 3, strong staining. We used the highest score for each sample. A score of 0 or 1 was defined as negative, whereas a score of 2 or 3 was defined as positive for the statistical analyses.

Table I. *Clinicopathologic parameters of the cancer cases examined*.

	median±SD (range)	
Age (y)	62.0±13.9 (27-84)	
	n	(%)
Gender		
Male	52	68.4
Female	24	31.6
Tumor size		
T1	8	10.5
T2	33	43.4
T3	16	21.1
T4	19	25.0
Nodal metastasis		
N0	49	64.5
N1-3	27	35.5
UICC stage		
I	8	10.5
II	28	36.8
III	12	15.8
IV	28	36.8
Histological type		
SCC well	34	44.7
SCC mod.	26	34.2
SCC poor	16	21.1
Smoking		
Smoker	49	64.5
Non smoker	27	35.5

The localization patterns of aPKCλ/ι were classified into two types: nuclear and cytoplasmic. All analyses were performed in at least three randomly photographed fields using the ×20 or ×40 objectives and ×10 eyepiece of the microscope.

Statistical analyses. Statistical analyses were performed using the JMP Pro program version 12.2 (SAS Institute Inc., Cary, NC). Clinicopathological parameters and the aPKCλ/ι expression or localization results were compared using the χ^2 test. Clinicopathological parameters included age at diagnosis (<60 *vs.* ≤60 years of age), sex (female *vs.* male), smoking history (nonsmoker *vs.* smoker), tumor diameter (<40 mm *vs.* ≥40 mm), nodal metastasis (negative *vs.* positive), pTNM stages (stagesI+II *vs.* stagesIII+IV, pathological type (well-differentiated *vs.* moderately differentiated *vs.* poorly differentiated), and tumor location (tongue *vs.* gingiva *vs.* other location). When the incidence was less than 5, χ^2 tests with Fisher's exact correction were applied. Two-sided probability values less than 0.05 were considered statistically significant. Kaplan–Meier curves were plotted to evaluate the association of aPKCλ/ι expression with 5-year overall survival rates (OS). The curves were compared using the log-rank test. Cox regression analysis was used to identify factors affecting 5-year survival rates.

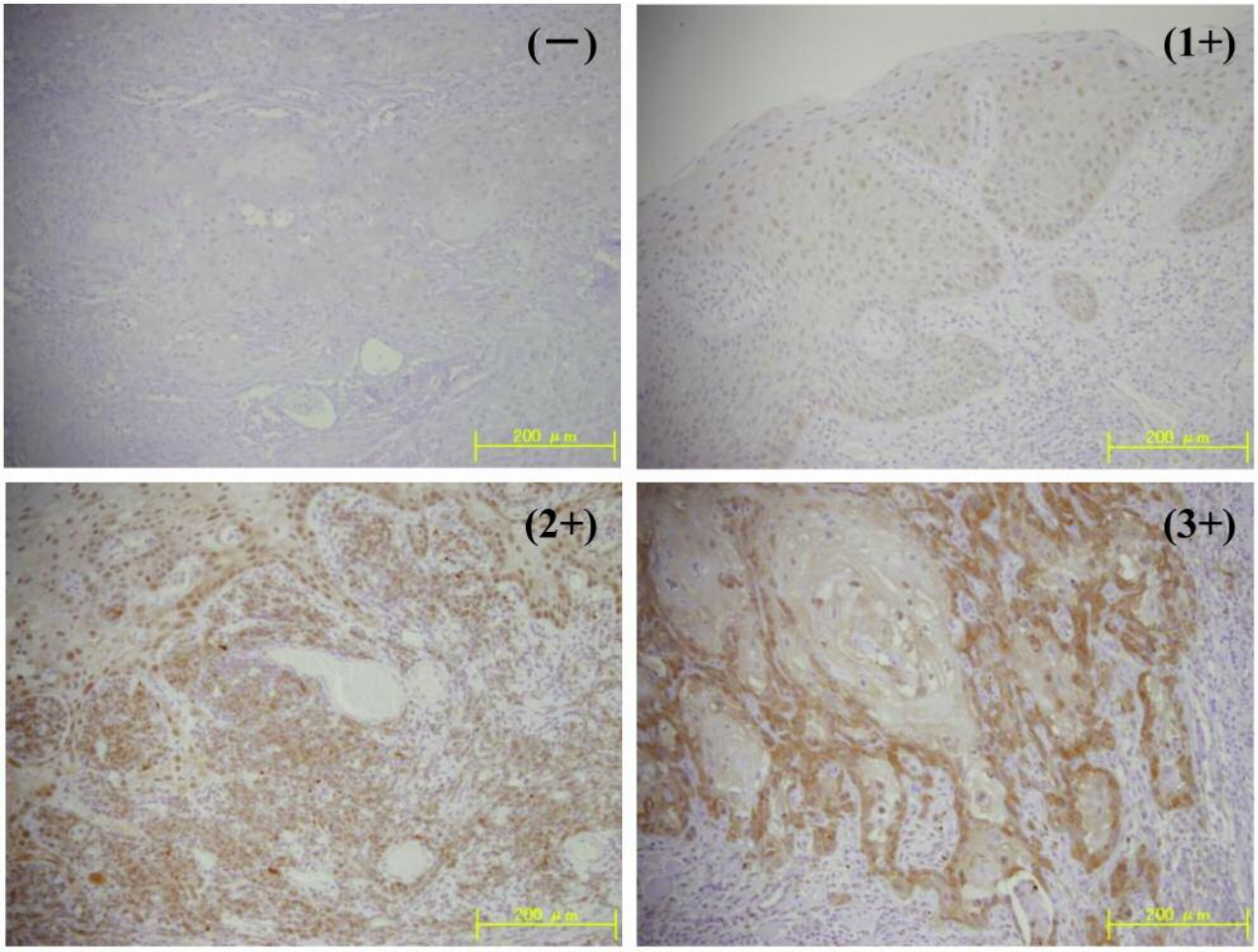

Figure 1. *The representative data of aPKCλ/ι immunostaining. -: no staining, 1+: weak to normal, 2+: moderate staining, 3+: strong staining. Score - and 1+ was defined as "negative", whereas 2+ and 3+ were defined as "positive" for the purposes of statistical analyses.*

Results

Patient characteristics. The clinicopathological features of the 76 patients are summarized in Table I. Patients included 24 women and 52 men ranging in age from 27 to 84 years (the average age, 62.0 years). Twenty-seven cases (35.5%) had nodal metastases. The clinical stages were distributed as follows: stage I, 8 (10.5%); stage II, 28 (36.8%); stage III, 12 (15.8%); stage IV, 28 (36.8%). The pathological types were distributed as follows: well-differentiated, 34 (44.7%); moderately differentiated, 26 (34.2%); poorly differentiated, 16 (21.1%). The median duration of follow-up was 67.3 months (range=3-132 months), and 5-year overall survival rate was 74.5%. The cause of death was due to present illness except for one patient who died from another reason.

Overexpression of aPKCλ/ι protein in OSCC. The expression of aPKCλ/ι protein was detected in 64 of the 76 (84.2%) cases. Semi-quantitative scoring was performed. Overexpression of aPKCλ/ι with a score of 2 or 3 was found in 28 cases (36.8%), whereas a score of 1 was found in 36 cases (47.4%) and a score of 0 (no detectable expression of aPKCλ/ι) in 12 cases (15.8%). Figure 1 shows representative images of each score for aPKCλ/ι expression. In normal oral mucosal tissue around the tumor, overexpression of aPKCλ/ι protein was not seen in any of the cases (0/31). Overexpression of aPKCλ/ι in OSCC was significantly higher than that of normal oral mucosa ($p<0.05$). Furthermore, the localization of aPKCλ/ι expression was nuclear type in 48 cases (63.2%) and cytoplasmic type in 16 cases (21.1%).

Correlation between the aPKCλ/ι expression pattern and the clinicopathological parameters associated with OSCC. Correlations between aPKCλ/ι expression and clinicopathological parameters are shown in Table II. The signal intensity of immunostaining for aPKCλ/ι was remarkably higher in poorly differentiated OSCC than in

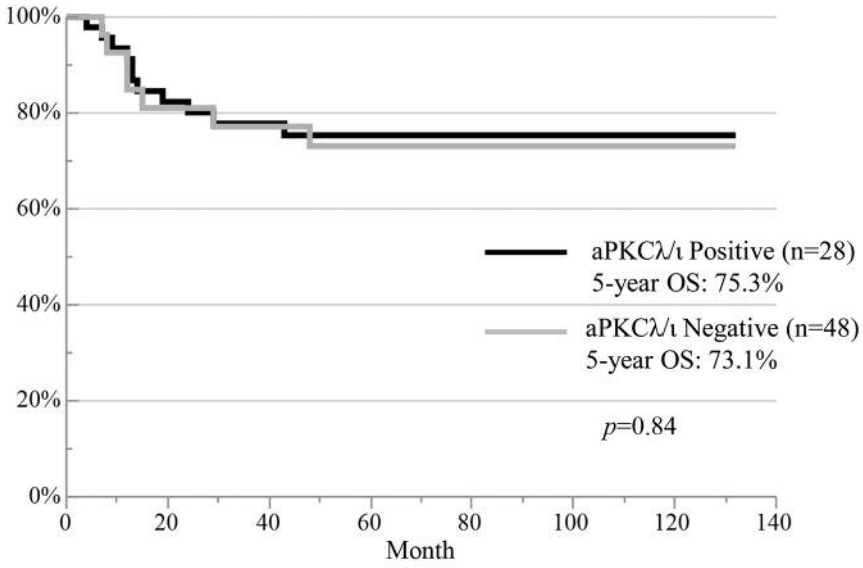

Figure 2. *Five-year overall survival rate and aPKCλ/ι expression. Black line indicates 5-year OS in aPKCλ/ι-positive group. Grey line shows 5-year OS in aPKCλ/ι-negative group. There was no significant difference between the two gruoups (p=0.84).*

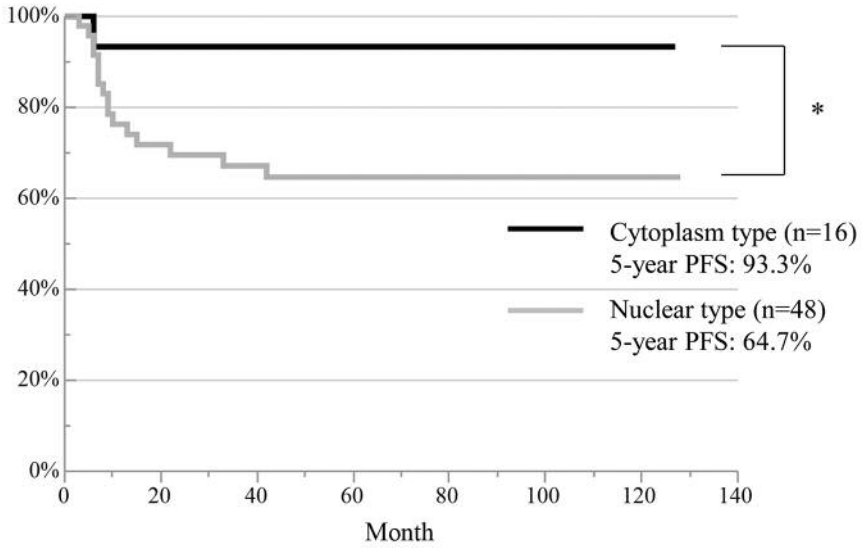

Figure 3. *Five-year progression-free survival rate and aPKCλ/ι location. Black line indicates 5-year PFS in the group of cytoplasmic localization of aPKCλ/ι. Grey line shows 5-year PFS in the group of nuclear locaton of aPKCλ/ι. There was significant difference between two groups (*p<0.05).*

moderately differentiated and well-differentiated OSCC ($p<0.01$). In addition, patients younger than 60 years of age showed higher intensity of immunostaining for aPKCλ/ι ($p<0.05$). Moreover, men with OSCC showed a higher expression level of aPKCλ/ι than women, according to immunostaining ($p<0.05$). Regarding advanced cases (stage III/IV, n=40), it is likely that higher aPKCλ/ι overexpression is accompanied by lymph node metastasis ($p=0.09$).

On the other hand, there was no correlation between aPKCλ/ι expression and tumor size, nodal metastasis, UICC classifications, tumor location, or recurrence rate. Furthermore, there was no significant difference in 5-year overall survival rates between aPKCλ/ι-positive (73.1%) and aPKCλ/ι-negative (75.3%) cases ($p=0.84$, Figure 2). In addition, there was no localization-specific aPKCλ/ι expression in cancer cells in OSCC ($p=0.112$, Table III), and

Table II. *Clinicopathological parameters and aPKCλ/ι expression.*

	aPKCλ/ι expression		*p*-Value
	2+, 3+ (28)	–, 1+ (48)	
Age (y)			
<60	15	12	0.015
≥60	13	36	
Gender			
Male	24	28	0.020
Female	4	20	
Tumor size			
T1-2	16	25	0.812
T3-4	12	23	
Nodal metastasis			
N0	16	33	0.331
N1-3	12	15	
UICC stage			
I•II	14	22	0.813
III•IV	14	26	
Histological type			
SCC well	7	27	0.004
SCC mod.	10	16	
SCC poor	11	5	
Smoking			
Smoker	21	28	0.214
Non smoker	7	20	

Table III. *aPKCλ/ι location and aPKCλ/ι expression.*

χ^2 test	aPKCλ/ι expression		*p*-Value
	2+ 3+	1+	
Location of aPKCλ/ι			
Nuclear	24	24	0.144
Cytoplasmic	4	12	

Table IV. *Clinicopathological parameters and aPKCλ/ι location.*

	aPKCλ/ι location		*p*-Value
	Nuc≥Cyt (48)	Nu<Cyt (16)	
Age (y)			
<60	21	5	0.558
≥60	27	11	
Gender			
Male	35	11	0.756
Female	13	5	
Tumor size			
T1-2	25	8	1.000
T3-4	23	8	
Nodal metastasis			
N0	29	9	0.777
N1-3	19	7	
UICC stage			
I•II	21	7	1.000
III•IV	27	9	
Histological type			
SCC well	21	7	0.981
SCC mod	16	5	
SCC poor	11	4	
Smoking			
Smoker	32	11	1.000
Non smoker	16	5	

Table V. *Clinical outcomes and aPKCλ/ι location.*

	Location of aPKCλ/ι		*p*-Value
	Nuc≥Cyt	Nuc<Cyt	
Overall survival (OS)	68.5%	86.7%	0.200
Progression-free survival (PFS)	64.7%	93.3%	0.049
Recurrence-free survival (RFS)	62.8%	87.1%	0.110
Locoregional control (LRC)	92.4%	100.0%	0.290

there was no statistical correlation between the localization of aPKCλ/ι protein and clinicopathological parameters (Table IV). As both the poor differentiation and younger ages of OSCC patients tend to lead to a worse prognosis (16-20), it is suggested that aPKCλ/ι overexpression may correlate with the malignancy of OSCC.

Risk factors affecting 5-year survival rate according to multivariate analysis. Multivariate analysis for 5-year overall survival was performed using logistic regression analysis with aPKCλ/ι expression, age, sex, pathological differentiation, stage, and smoking history, which are thought to be involved in the development of oral cancer, as covariates. As a result, stage had a significant impact on overall survival at a hazard ratio of 3.43. No significant difference was observed in other parameters. The odds ratio for aPKCλ/ι expression was 1.91, which was slightly higher in aPKCλ/ι-positive cases.

In sub-class analysis focusing on 66 cases in which aPKCλ/ι expression was observed, we investigated the relationship between some clinical outcomes and localization of aPKCλ/ι. The duration of median follow-up in the 66 patients was 66.4 months (range=3-132 months). As shown in Table V, there was no significant difference in 5-year overall survival rate. However, significant correlation was found between the localization of aPKCλ/ι (nuclear type: 64.7%, cytoplasm type: 93.3%, $p<0.05$) and progression-free survival rate (Figure 3).

Discussion

OSCC typically occurs in the elderly population with a peak incidence in the sixth and seventh decades of life (21). The median age of the cohort in this study was 62.0 years, similar to previous studies (16, 22). There has been considerable controversy in the literature regarding whether young patients with OSCC have a significantly worse prognosis than older patients. A number of reports supported that the younger OSCC patients tend to have a worse outcome than older patients (17, 18). Gravello *et al*. showed that patients younger than 40 years have a higher risk of recurrence and death than those older than 40 years (20). In contrast, other authors concluded that older patients with OSCC have a worse prognostic significance than younger patients (21). In this study, overexpression of aPKCλ/ι was found in patients younger than 60 years of age, but there was no statistically significant association between age and prognosis (data not shown).

Pathological tumor differentiation is thought to be critical as a factor associated with prognosis and resistance to chemotherapy and radiotherapy. A number of studies reported significant correlations between lower histological differentiation and poor prognosis (16, 19). In this study, overexpression of aPKCλ/ι protein was found to be remarkably higher in poorly differentiated OSCC compared with moderately differentiated and well-differentiated OSCC.

A previous study reported that aPKCλ/ι overexpression was correlated with clinical outcomes in gastric cancer (7). However, in this study, there was no correlation between 5-year survival rate and aPKCλ/ι expression in OSCC. The discrepancy between gastric cancer and OSCC might be because this study was conducted in a retrospective manner and used various treatment modalities. In fact, we employed different treatment methods as the stage of tumors increased, leading to better survival rates in the cases of stage III (8/9, 88.9%) and stage IV (14/24, 58.3%). However, in general, poorly differentiated cases and younger cases have worse prognoses. Therefore, aPKCλ/ι overexpression in OSCC might be correlated with prognosis.

A previous study demonstrated that aPKCλ/ι overexpression causes interleukin-6 (IL-6) autocrine action, which is involved in tumor growth in prostate cancer (23). In addition, the nuclear location of aPKCλ/ι was an independent risk factor for cervical intraepithelial neoplasia. In the oral cavity, leukoplakia is epithelial hyperplasia that is thought to be a pre-malignant disorder where a few cases develop OSCC. aPKCλ/ι immunostaining was performed in 4 specimens of leukoplakia cases, but aPKCλ/ι overexpression was not detected in any of the cases. Besides, the expression of IL-6 was not associated with aPKCλ/ι expression in OSCC (data not shown). Based on these findings, aPKCλ/ι may be overexpressed in OSCC during carcinogenesis from leukoplakia. Further investigation will be required to clarify this hypothesis in future.

Conclusion

A correlation was found between the overexpression of aPKCλ/ι and the degree of pathological differentiation in OSCC. The localization of aPKCλ/ι was associated with progression-free survival of OSCC. Expression and localization of aPKCλ/ι may be involved in the degree of malignancy in OSCC, which is related to prognosis.

Conflicts of Interest

The Authors state that they have no conflicts of interest to declare in regard to this study.

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