Short Communication

Increased Phospho-AKT (Ser^{473}) Expression in Bronchial Dysplasia: Implications for Lung Cancer Prevention Studies[1]

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Abstract

AKT, a downstream mediator of phosphatidylinositol 3′-kinase, is activated in non-small cell lung cancer (NSCLC), but we have not yet defined the stage of malignant transformation at which AKT is activated in the bronchial epithelium. We performed immunohistochemical analysis of activated AKT [phosphorylated (p)-AKT Ser^{473}] in tissue specimens of normal bronchial epithelium, bronchial hyperplasia and squamous metaplasia ("reactive" epithelium), bronchial dysplasia, and NSCLC. Among NSCLC specimens, immunohistochemical findings were correlated with patient demographics, tumor stage, histology, and survival. We observed p-AKT expression in 12 of 44 (27.3%) normal bronchial biopsy specimens, 4 of 9 (44.4%) reactive epithelium specimens, 22 of 25 (88%) dysplastic specimens, and 25 of 76 (33%) NSCLC specimens. Among patients with resected early-stage or locally advanced NSCLC, p-AKT expression had no effect on tumor stage, histology, or survival. Of the histological groups examined, bronchial dysplasia specimens expressed p-AKT most frequently, supporting AKT activation as an early event in lung cancer progression. Given its role as a mediator of malignant transformation, p-AKT should be investigated as a potential target in future lung cancer prevention studies.

Introduction

NSCLC[3] is the leading cause of cancer-related death in the United States (1). Approximately 80% of patients present with inoperable locally advanced or metastatic disease at diagnosis. The overall 5-year survival rate is approximately 12% (2). Given the poor outcome in patients with advanced disease, recent efforts have focused on the reversal of bronchial premalignancy. However, interventions made thus far in individuals at increased risk of developing lung cancer have revealed no benefit (3). Effectively reversing bronchial premalignancy will require that we identify the critical genetic and biochemical events that cause malignant progression in the bronchial epithelium.

Activation of the PI3K pathway causes malignant transformation *in vitro* and is sufficient to induce a variety of solid tumors in mouse models of human cancer (4–8). Factors contributing to PI3K pathway activation in cancer include alterations in the PI3K p85 regulatory subunit or the p110 catalytic subunit, PTEN, and AKT (also known as protein kinase B), which is a downstream effector molecule of the PI3K pathway. AKT is activated by the PI3K phospholipids PI-3,4,5-P3 and PI-3,4-P2, which bind to the pleckstrin homology domains of AKT and anchor it to the plasma membrane. In addition, the PI3K-dependent kinases phosphoinositide-dependent kinase and integrin-linked kinase activate AKT by phosphorylating it at Thr^{308} and Ser^{473}, respectively (9, 10). AKT activation is required for the transforming effects of PI3K (11).

Previous studies have reported increased expression of p-AKT (Ser^{473}) in NSCLC (12–17), providing evidence of AKT activation in fully transformed bronchial epithelium. However, it is not clear when AKT is activated during the process of malignant progression. Here we examined p-AKT (Ser^{473}) expression in normal bronchial epithelium, "reactive" epithelium (bronchial hyperplasia and squamous metaplasia), early bronchial neoplasia (bronchial dysplasia), and NSCLC. We found p-AKT (Ser^{473}) expression in a strikingly high percentage of bronchial dysplasia specimens, supporting PI3K pathway activation as an early event in lung tumorigenesis.

Materials and Methods

Tissue Specimens. We evaluated 44 normal bronchoscopic biopsy specimens and 9 specimens considered to have reactive histology (hyperplasia or squamous metaplasia) obtained at baseline from 20 former smokers who had at least a 20-pack-year smoking history and no evidence of cancer at the time of bronchoscopic examination.[4] We also evaluated 25 bronchoscopic biopsy specimens with dysplastic features obtained from 25 smokers who had at least a 30-pack-year smoking history and morphometric evidence of dysplasia in sputum samples (18) and 76 NSCLC specimens resected from 76 patients. Pathological staging of NSCLC specimens was based on op-

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; PI3K, phosphatidylinositol 3′-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; p-AKT, phosphorylated AKT.

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erative and pathological reports from clinical records at The University of Texas M. D. Anderson Cancer Center. Histological diagnosis of all specimens was confirmed by light microscopic analysis of formalin-fixed sections stained with H&E.

Immunohistochemical Staining. Immunohistochemical analysis of p-AKT was performed with a phosphospecific antibody (Ser^{473}) from Cell Signaling Technologies (Beverly, MA). Positive and negative controls for immunohistochemical staining were established with PTEN-null MDA-MD-468 and PTEN wild-type MCF-7 breast cancer cell lines, respectively. Preincubation of the phosphospecific antibody with phosphopeptides to Ser^{473} blocked immunohistochemical staining, as did deletion of the primary antibody from the staining procedure (data not shown), verifying the specificity of the antibody for p-AKT (Ser^{473}). Western blot analysis with the phosphospecific antibody also generated a single band of M_r 60,000 (data not shown), which is the expected size of p-AKT.

All tissue samples were fixed in formalin and embedded in paraffin. Bronchial biopsy tissue sections from each patient were placed on a single glass slide. NSCLC specimens were placed in a tissue microarray that included three core biopsies from each tumor block. The tissue samples were deparaffinized and rehydrated in three changes of xylene, three changes of 100% ethanol, and two changes of 80% ethanol. Antigen retrieval was performed with Dako Target Retrieval Solution (citrate buffer), and steam-heated for 25 min. The samples were then blocked for endogenous peroxidase with 3% solution hydrogen peroxide in 1× PBS with 0.1% sodium azide for 5 min, with Zymed avidin blocking solution for 15 min, with Zymed biotin blocking solution for 15 min, and then with Dako serum-free Protein Blocking Solution for 5 min. The slides were rinsed after each blocking procedure with 50 mM Tris-buffered saline containing 0.05% Tween 20 solution. Slides were incubated with anti-p-AKT (Ser^{473}) antibody (diluted 1:500) for 18 h at 4°C. Dako prediluted biotinylated universal secondary antibody from the LSAB+ kit was then applied for 15 min. After being rinsed with Tris-buffered saline, the slides were incubated for 15 min with Dako prediluted horseradish peroxidase-labeled streptavidin from the LSAB+ kit and then incubated for another 15 min with Biogenex diaminobenzidine solution. Dehydration in 95% ethanol and then in 100% ethanol was performed before the slides were cleared with xylene.

Bronchial biopsy specimens were examined by light microscopy and scored as positive for p-AKT expression if at least 10% of the epithelial cells had detectable staining. For the NSCLC tissue microarray specimens, BLISS slide scanner images of each core specimen were obtained and then scored. Biopsy samples were scored as positive if the specimen had focal or diffuse staining in the epithelial layer and detectable staining in at least 10% of the tumor cells. If any of the three core tumor block specimens stained positive, the tumor was considered to have positive p-AKT expression. The scores were then entered into Microsoft SQL database housed in the Department of Biostatistics and linked to patient medical record numbers via Active X (Bacus Laboratories). All data were encrypted to ensure patient confidentiality. Histology, tumor stage, and clinical outcomes were compared with biomarker staining.

Statistical Methods. Clinical and biological characteristics were analyzed for possible association with survival and recurrence by using univariate and multivariate Cox proportional hazard models. These characteristics included p-AKT expression, sex, smoking status and number of pack-years, prior cancer status, and tumor stage and histological type. Estimates of survival curves were derived from the Kaplan-Meier product-limit method and calculated from the time of surgery. Survival time comparisons between p-AKT-positive and p-AKT-negative groups were assessed by means of the log-rank test. Associations between categorical variables were evaluated by cross-tabulation and the χ^2 test. For continuous variables, mean differences between groups were assessed with the *t* test. All computations were carried out on a Dell PC with the Windows NT operating system in SAS with standard SAS procedures.

Table 1 Characteristics of patients who underwent bronchoscopic biopsies and tumor resection

	Normal[a] (n = 20)	Dysplasia (n = 15)	NSCLC (n = 76)
Age (yrs)			
Mean (±SD)	56.8 (9.3)	62 (6.7)	64.1 (9.1)
Median (range)	57.1 (38–72)	60 (49–72)	64 (42–88)
Gender			
Male	11 (55%)[b]	18 (72%)[b]	36 (47.4%)[b]
Female	9 (45%)	7 (28%)	40 (52.6%)
Ethnicity			
Caucasian	19 (95%)	22 (86.7%)	65 (84.2%)
Hispanic	0 (0%)	0 (0%)	6 (7.9%)
African-American	1 (5%)	0 (0%)	6 (7.9%)
Oriental	0 (0%)	1 (4%)	0 (0%)
Native American	0 (0%)	1 (4%)	0 (0%)
Unknown	0 (0%)	1 (4%)	0 (0%)
Smoker or former smoker	20 (100%)	25 (100%)	59 (77.6%)
Nonsmoker	0 (0%)	0 (0%)	18 (22.4%)
Pack-years			
Mean (±SD)	46.7 (26.1)	56.6 (21.4)	48.4 (38.8)
Median (range)	39.0 (20–120)	51 (30–111)	45.0 (0–140)

[a] This includes 44 normal specimens and 9 specimens considered to be hyperplasia or squamous metaplasia.
[b] Percentiles calculated within each histological group.

Results

Patient Characteristics. Table 1 summarizes the characteristics of patients who underwent bronchoscopic biopsies and NSCLC resection. The tissues analyzed from these patients included 44 normal specimens, 9 specimens with epithelial hyperplasia or squamous metaplasia, 25 dysplastic specimens, and 76 NSCLC specimens. Because hyperplasia and squamous metaplasia are considered reactive and potentially reversible histological abnormalities, we combined normal, hyperplasia, and squamous metaplasia into a single group (normal histology). The three histological groups (normal, dysplasia, and NSCLC) were well balanced for age, ethnicity, and smoking history, but not gender. Males accounted for 55%, 72%, and 47% of the normal, dysplasia, and NSCLC specimens, respectively. Table 2 summarizes the characteristics of the 76 resected NSCLCs, which included 29 adenocarcinomas, 33 squamous cell carcinomas, and 14 bronchoalveolar carcinomas. The majority of the tumors (96%) were early stage (I or II) by pathological staging; three others (one adenocarcinoma and two squamous cell carcinomas) had evidence of local invasion (T_4). When analyzed by histological groups, the tumors were well balanced for clinical stage.

p-AKT Expression in Normal, Premalignant, and Malignant Lung Tissues. We detected p-AKT expression in 12 of 44 (27.3%) normal specimens, 4 of 9 (44.4%) reactive specimens, 22 of 25 (88%) dysplastic specimens, and 25 of 76 (33%) NSCLC specimens (Fig. 1). The increased frequency of p-AKT

Table 2 Tumor characteristics

	Adenocarcinoma (n = 29)	Squamous cell (n = 33)	Bronchoalveolar (n = 14)	Total[a] (n = 76)
T				
1	13 (44.8%)[b]	10 (30.3%)[b]	6 (42.9%)[b]	29 (38.2%)
2	15 (51.7%)	20 (60.6%)	8 (57.1%)	43 (56.6%)
3	0 (0%)	1 (3.0%)	0 (0%)	1 (1.3%)
4	1 (3.5%)	2 (6.1%)	0 (0%)	3 (3.9%)
N				
0	28 (96.6%)	32 (96.9%)	14 (100%)	74 (97.4%)
1	1 (3.4%)	1 (3.1%)	0 (0%)	2 (2.6%)
2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
3	0 (0%)	0 (0%)	0 (0%)	0 (0%)
M				
0	29 (100%)	33 (100%)	14 (100%)	76 (100%)
1	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Stage				
IA	13 (44.8%)	9 (27.4%)	6 (42.9%)	28 (36.9%)
IB	14 (48.2%)	20 (60.6%)	8 (57.1%)	42 (55.3%)
IIA	0 (0%)	1 (3.0%)	0 (0%)	1 (1.3%)
IIB	1 (3.5%)	1 (3.0%)	0 (0%)	2 (2.6%)
IIIA	0 (0%)	0 (0%)	0 (0%)	0 (0%)
IIIB	1 (3.5%)	2 (6.0%)	0 (0%)	3 (3.9%)
IV	0 (0%)	0 (0%)	0 (0%)	0 (0%)

[a] Represents the sum of all histological subtypes.
[b] Percentiles calculated within each histological subtype.

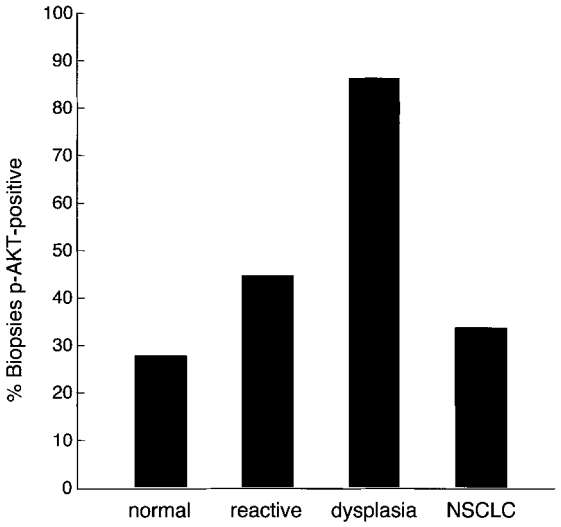

Fig. 1. Percentages of biopsy specimens that express p-AKT. Immunohistochemical analysis of p-AKT expression was performed on 44 bronchial biopsy specimens with normal histology, 9 specimens with reactive changes (hyperplasia or squamous metaplasia), 25 bronchial dysplasia specimens, and 76 NSCLC specimens. The percentages of specimens staining positively for p-AKT are illustrated.

expression in bronchial dysplasia specimens was statistically significant ($P < 0.001$, χ^2 test). Representative examples of p-AKT staining in normal epithelium, bronchial dysplasia, and NSCLC tissues are illustrated in Fig. 2. In the dysplastic bronchial epithelium and NSCLC cells, p-AKT was primarily cytoplasmic and, less frequently, nuclear (Fig. 3), which is consistent with the pattern of AKT expression reported in other tissues (19).

Among the patients with NSCLC, p-AKT expression was not associated with age ($P = 0.14$), gender ($P = 0.57$), race ($P = 0.43$), smoking status ($P = 0.81$), or number of pack-years of smoking ($P = 0.80$). With regard to tumor characteristics, p-AKT expression was not associated with tumor stage ($P = 0.27$), histological type ($P = 0.29$), or prior malignancy ($P = 0.94$). Therefore, we found no patient or tumor prognostic features that correlated with p-AKT expression in NSCLC.

Correlation of p-AKT Expression with Clinical Outcome. For the 76 patients with NSCLC, median follow-up after surgical resection was 104.9 months. Overall median survival was 96.5 months. p-AKT expression did not correlate with overall survival ($P = 0.40$) or time to recurrence ($P = 0.64$) in the 76 patients with NSCLC (univariate Cox proportional hazard model). Histological subset analysis showed no significant differences in survival between patients with p-AKT-positive *versus* p-AKT-negative tumors ($P = 0.29$, log-rank test). After adjusting for the effects of tumor stage and histology, p-AKT expression had no correlation with survival ($P = 0.54$) or time to recurrence ($P = 0.64$; multivariate Cox proportional hazard model).

Discussion

We found p-AKT (Ser^{473}) expression in a strikingly high percentage of bronchial dysplasia specimens. This is the first evidence, to our knowledge, of AKT activation in bronchial premalignancy. A growing body of evidence implicates the PI3K pathway in the development of human malignancies, including lung cancer (12–17). Findings from *in vitro* models indicate that activation of the PI3K pathway is sufficient to induce malignant transformation of human cells (4, 5). However, to date, studies on human tissues have not validated a role for the PI3K pathway in lung tumorigenesis. The increased expression of p-AKT we observed in bronchial dysplasia supports the hypothesis that the PI3K pathway is activated early in the process of lung tumorigenesis and may play a role in lung cancer progression. Moreover, our findings support evidence from colon adenomas showing that PI3K activation is an early event in sporadic colon carcinogenesis (20). However, we found no association of p-AKT expression with survival outcome in patients with NSCLC, a finding that stands in contrast to the adverse effect of p-AKT expression in gliomas (21). These findings suggest that the role of AKT in tumorigenesis is tissue specific.

Interestingly, we found that p-AKT (Ser^{473}) was expressed less frequently in NSCLC than in bronchial dysplasia. We propose several hypotheses to explain this finding. First, bronchial dysplasias are the malignant precursors of only a subset of histological subtypes of NSCLC, and premalignant lesions that lead to the remaining subtypes of NSCLC may not express p-AKT. Arguing against this hypothesis is our finding that p-AKT expression was not associated with a specific NSCLC histology. A second hypothesis is that AKT activation is required for the early stages of malignant transformation (to bronchial dysplasia) but not for progression to frank neoplasia. Implicit in this hypothesis is the assumption that clones of premalignant bronchial epithelial cells evolve during the process of malignant transformation, undergoing additional oncogenic events that render their survival independent of AKT. As one example of this process, breast cancer cells that initially express the estrogen receptor and depend on estrogen for survival can lose expression of that receptor, presumably becoming dependent on other growth factors for survival (22).

The genetic or biochemical events that contributed to the increase in p-AKT expression in bronchial dysplasia specimens have not yet been defined. As one potential candidate, recent

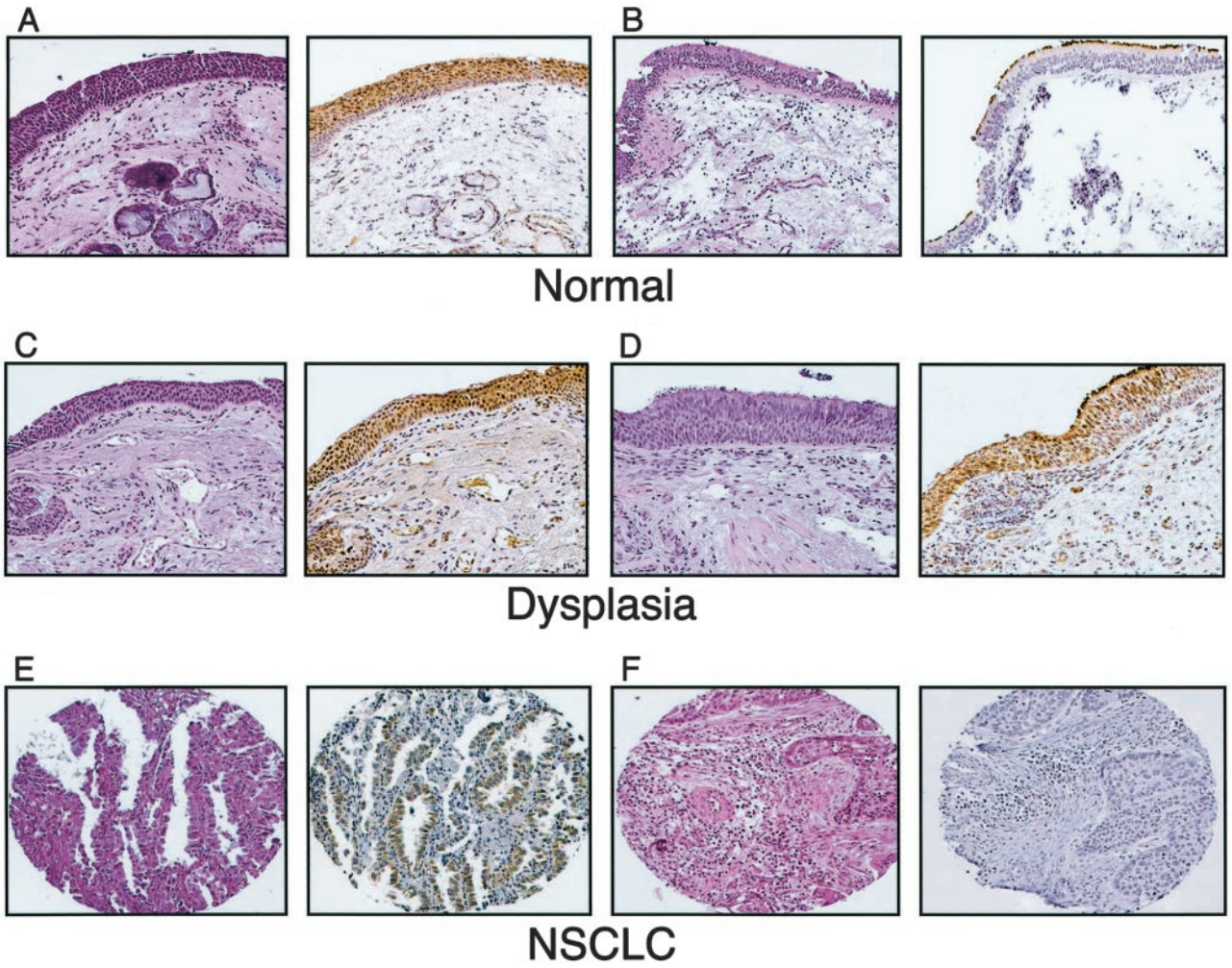

Fig. 2. p-AKT expression in (*A*) epithelial hyperplasia, (*B*) normal epithelium, (*C*) mild dysplasia, (*D*) moderate dysplasia, (*E*) adenocarcinoma, and (*F*) squamous cell carcinoma. Sections of each specimen were stained with H&E (*left panel*), immunostained for p-AKT (*right panel*), and photographed at ×20 magnification. The illustrated sections were interpreted as positive (*A*, *C*, *D*, and *E*) or negative (*B* and *F*) for p-AKT expression.

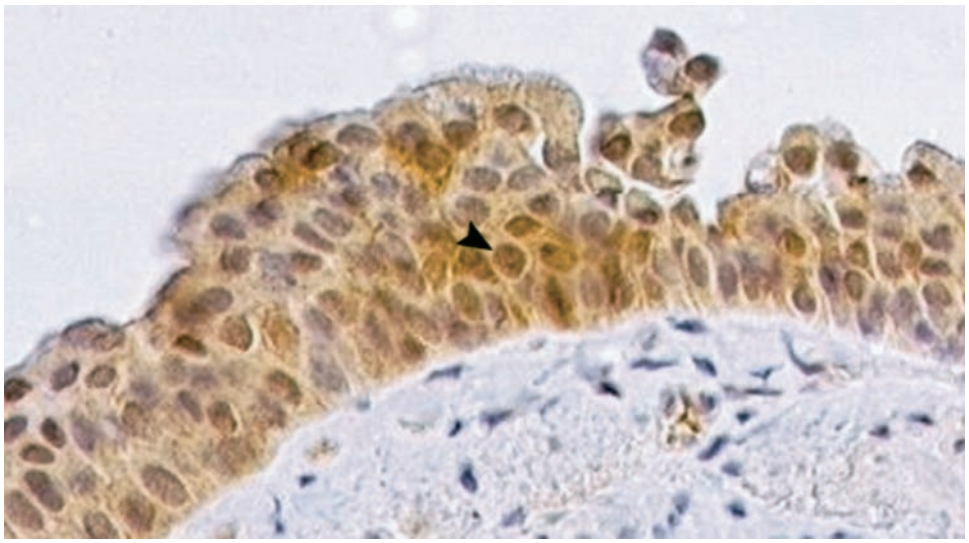

Fig. 3. p-AKT expression is primarily cytoplasmic and, less frequently, nuclear. A bronchial dysplasia specimen was subjected to immunohistochemical staining for p-AKT. A nucleus staining positively for p-AKT is indicated (*arrowhead*). The photograph was taken at ×20 magnification.

findings in NSCLC cells demonstrated amplification of a region of chromosome 3q that includes the p110 catalytic subunit of PI3K (17). Alternatively, AKT is activated through receptor tyrosine kinases, including epidermal growth factor receptor, which is important in lung cancer progression (23, 24). Agents that inhibit integrin-linked kinase and epidermal growth factor receptor are currently under development for the treatment of cancer (10, 25). Given the role of AKT in malignant transformation and the evidence presented here that AKT is activated in bronchial dysplasia, these agents should be considered in future lung cancer prevention studies.

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