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Original Article

Prognostic impact of high p16/cyclin D1 index in breast cancer

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Abstract: Proteins p16 and cyclin D1 (CCND1) are known to tightly regulate the G1/S transition during the cell cycle, but their role in breast cancer development and progression is not clear. We investigated 224 cases of breast cancer from the Kangbuk Samsung Medical Center between 2000-2005. Expression levels of p16 and CCND1 were assessed by tissue microarray-based immunohistochemistry. A p16/CCND1 index was divided into low- and high-expression groups using receiver operating characteristic curves. The p16/CCND1 index was significantly different across molecular subtypes and a high p16/CCND1 index was statistically correlated with survival rates. This p16/CCND1 index may be an indicator of poor patient outcome and thus, represents a potential therapeutic target.

Keywords: Breast cancer, p16, cyclin D1, index, prognosis

Introduction

Breast cancer is one of the most lethal diseases in women, but recent advances in treatment are improving patient outcomes. Various clinicopathological factors contribute to the development of treatments and are still being investigated. Based on DNA microarray analysis, breast cancers are subdivided into distinct subtypes that require treatment strategies differing in their use of drugs, treatment duration, and drug combinations [1-3]. Considering the diversity of breast cancers, genetic prognostic markers can improve the proper application and development of clinical treatments.

Many previous studies have researched single prognostic biomarkers related to clinicopathological factors and/or breast cancer patient outcomes [4-6]. Despite the convenience of single prognostic factors, their accuracy in evaluating patients' outcome and determining therapeutic strategies is limited. Therefore, applying a combination of molecular markers to predict prognosis could provide a more meaningful and reliable approach. Clinical application of combined molecular markers has recently been verified in breast cancer [7-9].

A series of highly ordered and tightly regulated cell cycle events lead to cell division. The G1 phase is a particularly important checkpoint that regulates cell division. G1 is the checkpoint when a cell commits to either continued cell division or exits the cell cycle and enters the quiescent stage called G0 [10, 11]. The G1 phase is followed by the S phase, when DNA is replicated and chromosomes are duplicated [12]. The regulation of the cell cycle is tightly controlled by various cell cycle factors. For example, p16 ($p16^{INK4a}$ or cyclin-dependent kinase inhibitor 2A) is a potent tumor suppressor protein that blocks the progression from G1 to S phase by inhibiting cyclin-dependent kinase 4 (CDK4)/cyclin D1 (CCND1) complex activity [13-15]. CDK4/CCND1 normally phosphorylates retinoblastoma protein (pRb), but its inhibition results in a hypo-phosphorylated form of pRb, which binds members of the E2F transcription factor and results in cell cycle arrest and transcription inhibition [16-18].

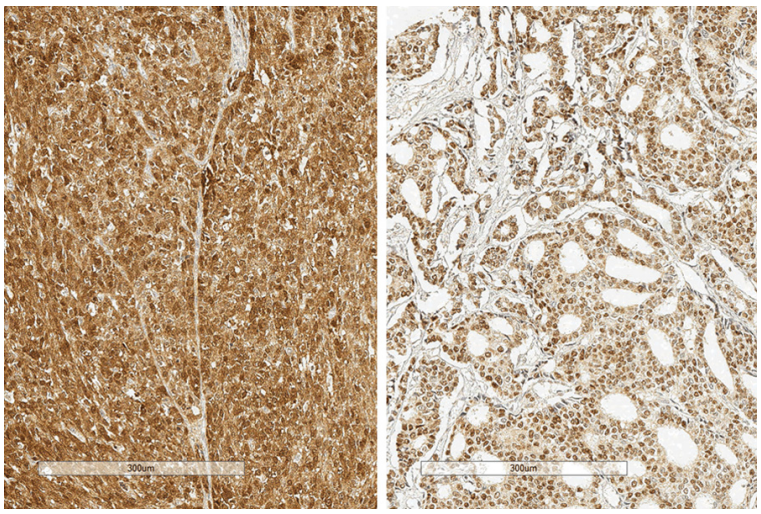

Figure 1. Immunohistochemical staining. Representative IHC for p16 (left) and CCND1 (right) in a breast cancer case with high p16 levels with respect to CCND1 (p16/CCND1 index > 4).

Overexpression of CCND1 and decreased expression of p16 are correlated with tumorigenesis and poor prognosis in various human cancers. In gallbladder cancer, Feng et al. reported low p16 expression levels and high CCND1 expression levels [19]. In laryngeal cancer, overexpression of CCND1 and decreased expression of p16 are associated with tumor development and metastasis to lymph nodes [20]. However, how CCND1 and p16 expression levels correlate with breast cancer is still controversial [21-25].

The aim of the present study was to analyze the prognostic value of p16 expression with respect to CCND1 expression (p16/CCND1 ratio) in a series of invasive breast cancer patients. We investigated whether the p16/CCND1 ratio could identify correlations with clinicopathological parameters and reflect patient outcomes.

Material and methods

Patient selection and characteristics

Clinicopathological data were collected from the medical records of 224 patients diagnosed with invasive ductal carcinoma at Kangbuk Samsung Medical Center between 2000-2005. Treatments for breast cancer included modified radical mastectomy in 203 patients and breast-conserving surgery with axillary lymph node dissection in 21 patients. The histological grade was determined according to the modified Bloom-Richardson-Elston grading system [26]. Tumors were staged with reference to their size and extension (T), regional lymph node involvement (N), and metastasis (M) using the 7th editionAJCC staging system. This study was approved by the Institutional Review Board of Kangbuk Samsung Hospital (Seoul, Korea). The Institutional Review Board waived the need for consent in this study (KBSMC 2017-07-037).

Tissue microarray construction

A series of tumor tissue microarray (TMA) specimens were assembled using a tissue array instrument (AccuMac Arrayer; ISU ABXIS Co. Ltd., Seoul, Korea). Tumor TMAs consisted of 10 × 6 arrays of 2.0 mm tissue cores from representative paraffin blocks. Taking into account the limitations associated with selecting representative areas of tumors, we used duplicate tissue cores of 2.0 mm diameter from each donor block. The percentage of tumor in the tissue cores was > 70%.

Immunohistochemical staining

All immunohistochemistry (IHC) was performed with formalin-fixed, paraffin-embedded tissue sections. Briefly, 5-μm-thick sections were obtained with a microtome, transferred onto adhesive slides, and dried at 62°C for 30 minutes. After incubation with primary antibodies, immunodetection was performed with biotinylated anti-mouse immunoglobulin, followed by peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3,3'-diaminobenzidine chromogen as the substrate. The primary antibody incubation step was omitted in the negative control. Positive control tissue was used per the manufacturer's recommendation. Slides were counterstained with Harris hematoxylin.

Immunostaining with antibodies against human epidermal growth factor receptor 2 (HER2, 1:200; SP3 Clone; Labvision, Fremont, CA, USA), estrogen receptor (ER, clone SP1, 1:200,

Table 1. Correlation between clinicopathologic parameters and p16/CCND1 index

Parameter	N = 224	p16/CCND1 index in tumor		*p* value (χ^2 test)
		Low (n = 161)	High (n = 63)	
Age				
< 45 years old	87	54 (33.5%)	33 (52.4%)	**0.009**
≥ 45 years old	137	107 (66.5%)	30 (47.6%)	
T category				
T1	84	65 (40.4%)	19 (30.2%)	0.062*
T2	125	88 (54.7%)	37 (58.7%)	
T3	15	8 (5%)	7 (11.1%)	
N category				
N0	100	70 (43.5%)	30 (47.6%)	0.672*
N1	70	52 (32.3%)	18 (28.6%)	
N2	26	18 (11.2%)	8 (12.7%)	
N3	28	21 (13%)	7 (11.1%)	
Tumor size				
≤ 2 cm	101	79 (49.1%)	22 (34.9%)	**0.056**
> 2 cm	123	82 (50.9%)	41 (65.1%)	
Tumor border				
Well-defined	43	26 (16.1%)	17 (27%)	0.064
Ill-defined	181	135 (83.9%)	46 (73%)	
Number of tumors				
Single	209	152 (94.4%)	77 (90.5%)	0.371†
Multiple	15	9 (5.6%)	6 (9.5%)	
Histologic grade				
1	32	25 (15.5%)	7 (11.1%)	**0.004***
2	107	86 (53.4%)	21 (33.3%)	
3	85	50 (31.1%)	35 (55.6%)	
Lymphatic invasion				
Negative	109	81 (50.3%)	28 (44.4%)	0.43
Positive	115	80 (49.7%)	35 (55.6%)	
Vascular invasion				
Negative	207	150 (93.2%)	57 (90.5%)	0.575
Positive	17	11 (6.8%)	6 (9.5%)	
Perineural invasion				
Negative	189	135 (83.9%)	54 (85.7%)	0.73
Positive	35	26 (16.1%)	9 (14.3%)	
Tumor necrosis				
Absence	131	104 (64.6%)	27 (42.9%)	**0.003**
Presence	93	57 (35.4%)	36 (57.1%)	
ER				
Negative	73	35 (21.7%)	38 (60.3%)	**< 0.001**
Positive	151	126 (78.3%)	25 (39.7%)	
PR				
Negative	101	60 (37.3%)	41 (65.1%)	**< 0.001**
Positive	123	101 (62.7%)	22 (34.9%)	
HER2				
Negative	164	124 (77%)	40 (63.5%)	**0.04**
Positive	60	37 (23%)	23 (36.5%)	

CCND1, cyclin D1; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. *linear by linear association test. †Fisher's exact test. $P < 0.05$ is shown in bold.

Labvision, Fremont, CA, USA), progesterone (PR, clone Pg-R636, 1:200, Dako, Glostrup, Denmark), and Ki-67 (clone MIB-1, 1:500, Dako, Glostrup, Denmark) was performed using a Dako Autostainer with a Universal Staining System (Dako-Cytomation, Carpinteria, CA, USA) and a ChemMate TM DAKO EnVision TM Detection kit.

Standardized staining protocols were provided by Ventana for the CINtec p16 Histology kit (MTM LaboratoriesInc,WestboroughMassachusetts) and rabbit CCND1 monoclonal antibody (RM-9104-S0, 1:100, Neomarkers) was used.

Interpretation of p16/CCND1 index

The values of p16 and CCND1 were evaluated in the hot spot area (**Figure 1**). Expression was graded according to both the intensity and percentage of positively stained tumor cells. The intensity of staining (p16, cytoplasmic and nuclear stain; CCND1, nuclear stain) was recorded separately as follows: 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong). The proportion of staining was graded as follows: 0 (0-5%), 1 (6-25%), 2 (26-50%), 3 (51-75%), or 4 (> 75%), and the immunoreactive score (IRS) was calculated (intensity × proportion). We evaluated the average IRS of two cores in tumor samples.

The relative index formula was as follows: p16/CCND1 index = p16 IRS - CCND1 IRS. The calculated values were subsequently divided into two groups by receiver operating characteristic (ROC) curves, which were used to evaluate the relationship between patient death and p16/CCND1 index. The ROC curve showed less predictive power for

Table 2. Expression of p16/CCND1 index according to molecular subtype

p16/CCND1 index	Luminal A	Luminal B HER2-	Luminal B HER2+	HER2+	Triple-negative	*p* value
Low	99 (85.3%)	5 (62.5%)	22 (78.6%)	15 (46.9%)	20 (50%)	**< 0.001***
High	17 (14.7%)	3 (37.5%)	5 (21.4%)	17 (53.1%)	20 (50%)	
Total no.	116	8	28	32	40	

CCND1, cyclin D1; HER2, human epidermal growth factor receptor 2. *Comparison of p16/Cyclin D1 index between luminal A and B versus HER2 and triple-negative. $P < 0.05$ shown in bold.

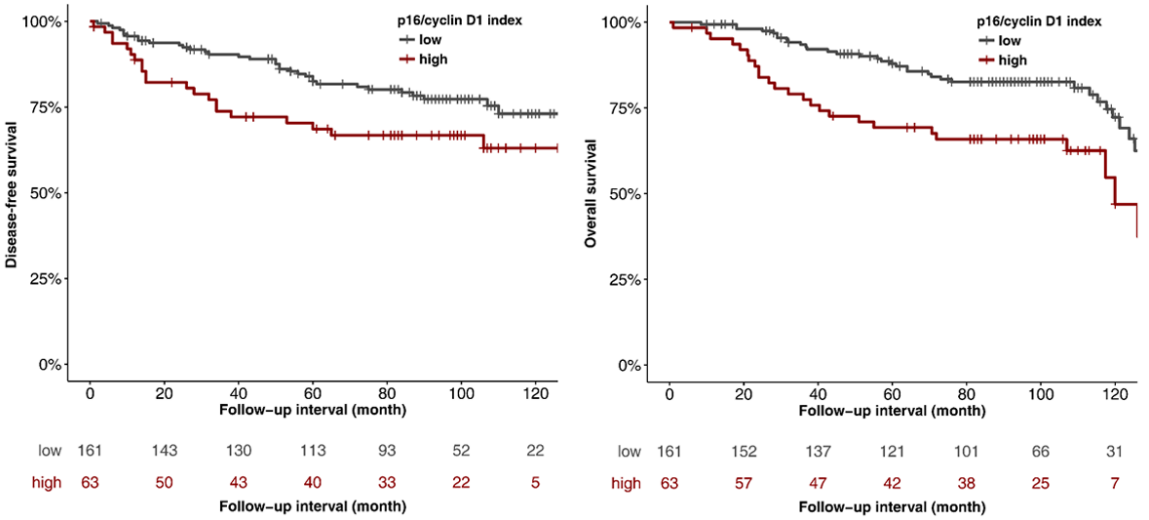

Figure 2. Disease-free and overall survival curves derived by the Kaplan-Meier method showing correlation with the p16/CCND1 index according to all cases (all P < 0.050).

correlating overall survival (OS) with p16/CCND1 index (area under the ROC curve = 0.549). The optimal cut-off value was 4. The p16/CCND1 index was classified as low (index ≤ 4) and high (index > 4).

Tumor phenotype classification

In this study, we classified breast cancer phenotypes according to the IHC results for ER, PR, HER-2, Ki-67, and FISH results for HER-2 as follows [27, 28]: luminal A (ER+ and/or PR+, HER2-, Ki-67 < 14%), luminal B HER2- (ER+ and/or PR+, HER2-, Ki-67 ≥ 14%), luminal B HER2+ (ER+ and/or PR+, HER2+, any Ki-67), HER2+ (ER- and PR-, HER2+), and triple-negative (ER-, PR-, and HER2-).

Statistical analysis

Categorical variables were compared using the Chi-square/Fisher's exact and linear-by-linear association tests. For the survival analyses, plots were generated using the Kaplan-Meier curve, and were compared using the log-rank test. Multivariate analysis was performed to identify independent prognostic markers for OS and disease-free survival (DFS) using a Cox multistep regression model. A value of $P < 0.05$ was considered significant. All statistical analyses were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinicopathological characteristics associated with p16/CCND1 index

Complete results of p16 and CCND1 IHC stains and survival data were obtained from 224 female patients with a median age of 47 years (range, 25-79 years). Other clinicopathological characteristics are provided in **Table 1**.

A total of 161 (71.9%) patients exhibited low p16/CCND1 index and 63 (28.1%) patients exhibited high p16/CCND1 index. High p16/CCND1 index was statistically associated with young age (P = 0.009) and worse clinicopathological characteristics, such as high histologic

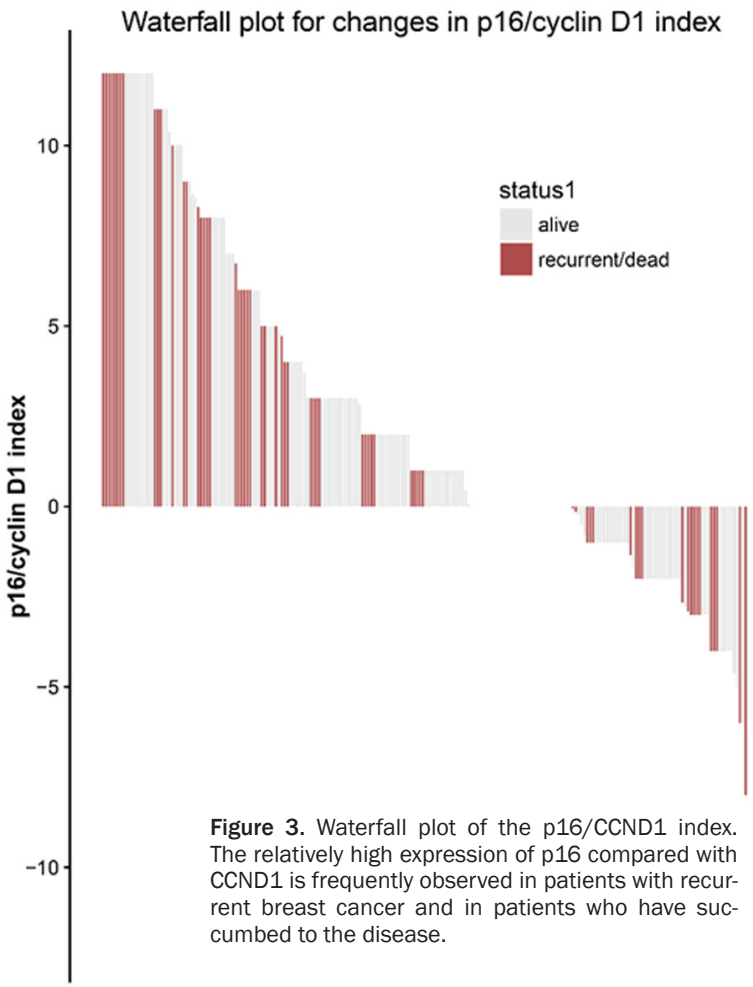

Figure 3. Waterfall plot of the p16/CCND1 index. The relatively high expression of p16 compared with CCND1 is frequently observed in patients with recurrent breast cancer and in patients who have succumbed to the disease.

grade (P = 0.004), tumor necrosis (P = 0.003), ER negativity (P < 0.001), PR negativity (P < 0.001), and HER2 positivity (P = 0.040).

p16/CCND1 index according to molecular subtypes

The most frequent molecular subtype was luminal A, found in 116 patients (**Table 2**). The frequency of the other subtypes was as follows: luminal B HER2- (8 patients); luminal B HER2+ (28 patients); HER2+ (32 patients); and triple-negative (40 patients). In patients with a high p16/CCND1 index, the distribution of subtypes was as follows: luminal A (17 patients); luminal B HER2- (3 patients); luminal B HER2+ (5 patients); HER2+ (17 patients); and triple-negative (20 patients). Patients were divided into two groups (luminal A or B versus HER2+ or triple-negative), and a significantly higher p16/CCND1 index in the HER2+/triple-negative group was observed (P < 0.001).

Comparison between survival based on p16/CCND1 index

A high p16/CCND1 index was significantly correlated with poor DFS and OS (P < 0.05) (**Figure 2**). The outcome of the 224 patients is shown in a waterfall plot (**Figure 3**). A high p16/CCND1 index was frequently noted in patients who had undergone recurrence or died from breast cancer. Other histological parameters such as AJCC stage, histologic grading, ER/PR status, lymphatic invasion, vascular invasion, and perineural invasion were also correlated with worse DFS or OS (P < 0.05).

After adjusting for confounders like the histological parameters, significant relationships were found between the p16/CCND1 index and OS (HR, 1.850; 95% CI, 1.005-3.243; P = 0.032) (**Table 3**).

Discussion

Our assessment using the p16/CCND1 index in breast cancer showed a statistical correlation between high p16/CCND1 index and poor prognostic parameters, such as high histologic grade, tumor necrosis, ER negativity, PR negativity, and HER2 positivity, in concordance with previous studies [22, 24, 29, 30]. According to the molecular subtypes, a high p16/CCND1 index was more frequently detected in HER2+ and triple-negative breast cancers than in luminal type cancers. The inverse relationship between p16/CCND1 index and ER/PR status in our study could be explained by the fact that high p16 and low CCND1 levels can induce estrogen-independent proliferation of breast cancer cells [29]. With the increasing use of hormonal therapy for patients with breast cancer, further investigation will be needed to define the exact mechanisms responsible for this relationship.

During the development and progression of malignant neoplasms, previous literature has

Table 3. Disease-free and overall survival analyses correlated with p16/CCND1 index

Disease-free survival	Univariate significance*	Multivariate significance†	Hazard ratio	95% CI
p16/CCND1 index (low vs. high)	**0.047**	0.164	1.545	0.837-2.852
AJCC stage (I or II vs. III)	**< 0.001**	**0.012**	2.103	1.178-3.754
Histologic grade (1 or 2 vs. 3)	**< 0.001**	0.294	1.424	0.736-2.757
ER/PR status (negative vs. positive)	**0.011**	0.831	0.931	0.480-1.803
Lymphatic invasion (absence vs. presence)	**< 0.001**	0.411	1.339	0.668-2.683
Vascular invasion (absence vs. presence)	**< 0.001**	**0.001**	4.094	1.782-9.405
Perineural invasion (absence vs. presence)	**< 0.001**	0.098	1.855	0.893-3.855
Overall survival				
p16/CCND1 index (low vs. high)	**0.002**	**0.032**	1.85	1.005-3.243
AJCC stage (I or II vs. III)	**0.001**	0.054	1.735	0.991-3.04
Histologic grade (1 or 2 vs. 3)	**< 0.001**	0.051	1.815	0.996-3.308
ER/PR status (negative vs. positive)	**< 0.001**	0.429	0.787	0.434-1.425
Lymphatic invasion (absence vs. presence)	**< 0.001**	0.642	1.172	0.6-2.288
Vascular invasion (absence vs. presence)	**< 0.001**	**< 0.001**	5.102	2.049-12.709
Perineural invasion (absence vs. presence)	**< 0.001**	0.502	1.314	0.592-2.918

CCND1, cyclin D1; ER/PR status, estrogen and/or progesterone receptor. *log rank test. †Cox proportional hazard model; adjusted for AJCC stage, histologic grade, ER/PR status, lymphatic/vascular/perineural invasion. $P < 0.05$ is shown in bold.

reported that the cell cycle is altered [11, 13, 19, 31, 32]. Similar to other cancers, breast cancer has altered p16 function through promoter methylation and the overexpression of CCND1 is associated with tumor progression to malignancy [33, 34]. Peurala et al. reported that patients with high expression of p16 and CCND1 in cancer cells showed better prognosis [23]. However, other studies have also found associations between high expression level of p16 and/or CCND1 and poor patient outcome [21, 29, 35, 36]. We assumed that these conflicting results may derive from the limitation of single molecular marker analysis. This could be resolved by applying a combination of molecular markers since cell proliferation is regulated by a complex interplay of cellular substrates. Our present study demonstrates that the high p16/CCND1 index has a superior prognostic value than that of single markers.

High p16/CCND1 index that showed a significant correlation with DFS (P = 0.047) or OS (P = 0.002) was independently associated with poor OS rate (HR, 1.850; 95% CI, 1.005-3.243; P = 0.032) after multivariate adjustment for other variables. Since p16 overexpression is identified mainly in tumors with dysfunctional pRb [21, 37, 38], high p16 expression may be indicative of pRb inactivation, which can lead to cell cycle arrest. The suppression of cell cycle progression by p16 is through the regulation of pRb [39]. Moreover, the expression level of Ki-67, a known proliferation index for malignant tumors, was significantly higher in p16-positive triple-negative breast carcinomas [40]. This could indicate that p16 is involved in tumor progression. However, the number of triple-negative cancers in this study was insufficient to implicate a correlation between Ki-67 and p16 expression levels.

In the present study, we found that the p16/CCND1 index had a better prognostic value in breast cancer, and that it was associated with aggressive clinicopathologic parameters. However, there are some limitations to these results that must be taken into consideration. First, other molecules involved in the p16-CCND1/CDK4-pRb pathway should be comprehensively investigated to improve the understanding of the complex interactions regulating the cell cycle. Second, a large-sized study using a continuous p16/CCND1 index could prevent unintentional loss of information compared with dichotomizing two groups (low- and high-expression). The cut-off value is controversial due to the variable length of follow-up or treating survival.

In summary, this study shows that the p16/CCND1 index is different across the molecular subtypes and is statistically correlated with survival rates. Therefore, the p16/CCND1 index

can be an indicator of poor patient outcomes and can serve as a potential therapeutic target.

Disclosure of conflict of interest

None.

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