Human Pathology (2019) **83**, 7–13

ELSEVIER

Human PATHOLOGY

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Original contribution

Breast cancers with a *HER2/CEP17* ratio of 2.0 or greater and an average *HER2* copy number of less than 4.0 per cell: frequency, immunohistochemical correlation, and clinicopathological features☆,☆☆

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Received 26 June 2018; revised 25 July 2018; accepted 1 August 2018

Keywords:
Breast cancer;
Human epidermal growth factor receptor 2;
Fluorescence in situ hybridization;
Centromere enumeration probe for chromosome 17;
Chromosome 17 monosomy

Summary The 2013 American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP) guidelines classified breast cancers with a fluorescence in situ hybridization dual-probe *HER2/CEP17* ratio of 2 or greater as "amplified," inclusive of cases with a *HER2* copy number less than 4. The 2018 ASCO/CAP update assigns *HER2*/neu status for the latter group in a fashion that is highly dependent on the associated immunohistochemical findings. Herein, the authors define the frequency, immunohistochemical correlates, and other clinicopathological features of breast cancers with *HER2/CEP17* ratio of 2 or greater and *HER2*/neu copy number less than 4 (group A), based on an analysis of an institutional cohort assessed for *HER2*/neu status by both florescence in situ hybridization and immunohistochemistry and scored using 2013 ASCO/CAP criteria. Group A cases were compared with a group B of *HER2*/neu-amplified breast cancers with a *HER2*/neu copy number of 4 or greater regarding a variety of clinicopathological features. One hundred sixty-nine (14%) of 1201 cases were *HER2*/neu amplified, 18 (10.7%) in group A and 151 (89.3%) in group B. By immunohistochemistry, 61.1% of group A cases were *HER2*/neu negative, 7 (38.9%) were equivocal, and none were positive. In contrast, 66.9% of group B cases were *HER2* positive (3+). We could not demonstrate statistically significant differences between the 2 groups regarding standard clinicopathological variables. In summary, our group A cases account for 1.5% of breast cancers, and 10.7% of all *HER2*/neu-amplified cancers classified as such based on 2013 ASCO/CAP criteria. They are predominantly *HER2*/neu negative by immunohistochemistry, which suggests that they are biologically different from classically *HER2*/neu-amplified cases and which validates the 2018 ASCO/CAP guideline against automatically classifying such cases as *HER2*/neu amplified.

☆ Disclosures: None.

☆☆ This work was presented in part at the Annual Meeting of the United States and Canadian Academy of Pathology, Vancouver, Canada, March 2018.

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https://doi.org/10.1016/j.humpath.2018.08.005
0046-8177/

1. Introduction

The human epidermal growth factor receptor type 2 (*HER2/neu or HER2*) gene is located on the long arm of chromosome 17 (17q12) and encodes a transmembrane receptor tyrosine kinase protein. *HER2* amplification and consecutive receptor overexpression in breast cancers are well known to have prognostic and predictive significance [1-4]. However, *HER2*-directed therapy is associated with significant costs and potential toxicities [5].Therefore, it is of paramount clinical significance to accurately determine the *HER2* status of breast cancers, so that only patients who are most likely to benefit from *HER2*-directed treatments are so treated [5-7].

The American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) have periodically issued detailed guidelines and updates for conducting and interpreting *HER2* testing, which may include immunohistochemistry (IHC) and/or in situ hybridization, to standardize the performance and reliability of *HER2* testing across laboratories [5,8]. In dual-probe fluorescence in situ hybridization (FISH) assays, the *HER2* copy number is typically reported relative to a chromosome enumeration probe 17 (*CEP17*) in a ratio [5,8].

The 2013 ASCO/CAP updated guidelines for the interpretation of *HER2* amplification in breast cancers call for the classification of cases with a FISH dual-probe *HER2*/*CEP17* ratio of 2 or greater as *amplified,* irrespective of *HER2* copy number. Therefore, cases with mean *HER2* signals/cell of less than 4 and *HER2*/*CEP17* ratio of 2 or greater are reported as *HER2* positive [5]. This pattern frequently occurs in the setting of low mean *HER2* copy number (<4) and loss of chromosome 17 copy number due to true monosomy (loss of a chromosome) or loss of a portion of the chromosome 17 [9,10]. The 2013 ASCO/CAP guideline of classifying cases with a *HER2*/*CEP17* ratio of 2 or greater and a copy number of less than 4 as *HER2* amplified was largely based on a single clinical trial [11], was controversial even from its inception, and there has been a lack of consensus on the validity of this approach [5,12]. Based on subsequently published works [10,13-15], an update to the 2013 ASCO/CAP guidelines has recently been published [16]. According to these 2018 ASCO/CAP guidelines, cases with a *HER2*/*CEP17* ratio of 2 or greater and a copy number of less than 4 would no longer be automatically classified as *HER2* amplified [16]. Rather, their final assigned *HER2* status will be highly dependent on the IHC findings: IHC-negative and IHC-positive cases will be classified as *HER2* negative and *HER2* positive, respectively; IHC-equivocal cases will need to have the FISH slide recounted by an observer that is blinded to prior results, counting at least 20 cells. If *HER2*/*CEP17* ratio is still at least 2 and a copy number is still less than 4 after this recount, the case should be classified as *HER2* negative with a comment. If the FISH category changes, such cases would be "adjudicated per internal procedures to determine the final category" [16].

There are relatively limited data regarding the clinicopathological profile of the breast cancers with mean *HER2* copies per cell of less than 4 and *HER2*/*CEP17* ratio of 2 or greater. Exploring the clinicopathological characteristics of this group of tumors could potentially provide some insights into whether they are truly similar to *HER2*-amplified breast cancers with at least 4 *HER2* copies and *HER2*/*CEP17* ratio of 2 or greater. At our institution, *HER2* is performed by FISH and IHC on all cases of breast cancer [17], which presented an opportunity to retrospectively assess the 2018 ASCO/CAP guidelines on this specific cohort. The aim of this study is to examine the frequency, immunohistochemical correlates, and other clinicopathological features of breast cancers with mean *HER2* signals/cell of less than 4 and *HER2*/*CEP17* ratio of 2 or greater.

2. Materials and methods

After approval from the Human Research and Protections Program at the University of California San Diego (no. 17205), consecutive cases of primary and metastatic breast carcinoma that underwent *HER2* testing at our institution between

Table 1 Clinicopathological characteristic of the breast cancers

Tumor characteristics	Value
Tumors types	
IDC	822 (74%)
Invasive lobular carcinoma	101 (9%)
Mixed ductal and lobular	128 (11.5%)
Special types	61 (5.5%)
NA	89
Histologic grade	
G1	190 (17%)
G2	587 (53%)
G3	333 (30%)
NA	91
Pathologic stage	
T1	542 (58%)
T2	306 (33%)
T3	85 (9%)
NA	268
Lymph node status	
Positive lymph nodes	366 (39%)
Negative lymph nodes	576 (61%)
NA	259
Hormone status	
ER+	1012 (84%)
ER−	189 (16%)
PR+	889 (74%)
PR−	312 (26%)
HER2 IHC	
Negative (0, 1+)	765 (64%)
Equivocal (2+)	322 (27%)
Positive (3+)	114 (9%)
HER2 FISH (*HER2*/*CEP17*)	
Positive	169 (14%)
Equivocal	122 (10%)
Negative	910 (76%)

Abbreviations: G, grade; NA, not available; T, tumor stage.

January 2014 and May 2017 were identified by searching an electronic database. In all cases, *HER2* testing was performed by both IHC and FISH methods, as is our institutional protocol [17]. Patient demographic data as well as tumor type, tumor stage (American Joint Committee on Cancer 2009) and grade (Elston-Ellis modification of Scarff-Bloom-Richardson grading system), hormone receptor status, *HER2* IHC results, and *HER2* FISH results were recorded from pathology reports; there was no renewed review of slides. All aspects of specimen handling, including minimal and maximal fixation times, as well as cold ischemic times, followed CAP or 2013 ASCO/CAP guidelines [5]. This cohort formed the basis of a previously reported study on the use of alternative probes for *HER2*/neu determination in breast cancer [18].

2.1. *HER2* status determination by IHC

Formalin-fixed, paraffin-embedded 4-μm-thick sections of tissue were used for IHC staining. *HER2* IHC was performed using a Ventana automated platform (Benchmark ULTRA; Ventana, Tucson, AZ). IHC assay for *HER2* (rabbit monoclonal antibody, clone 4B5) was carried out by following the manufacturer's instruction and using appropriate controls. Results for case were interpreted and reported using ASCO/CAP 2013 guidelines by 1 of 6 staff pathologists [5].

2.2. *HER2* status determination by FISH

Formalin-fixed, paraffin-embedded 4-μm-thick sections of tissue were used for FISH testing. Hematoxylin and eosin (H&E)–stained sections were evaluated by a pathologist to label the invasive carcinoma. FISH analyses were performed using dual-color *HER2*/*CEP17* assay (PathVysion Probe Kit; Abbott Molecular, Abbott Park, IL). Fluorescence hybridization signals were analyzed and captured using CytoVision software (Leica Biosystems Richmond, Inc, Richmond, IL). *HER2* and *CEP17* signals were manually counted by 2 technologists, each individually scored 20 tumor cell nuclei and calculated independent *HER2*/*CEP17* ratios. If their counts are comparable, an average is used to determine the final copy numbers. If results are significantly discrepant, the counts are repeated. Results were interpreted by a pathologist and a cytogeneticist and were reported using ASCO/CAP 2013 criteria [5].

2.3. Statistical analysis

Clinicopathological features of the tumors were compared between *HER2*/neu-amplified cases with a *HER2*/*CEP17* ratio of 2.0 or greater, average *HER2*/neu copy number less than 4.0 signals/cell (group A), and *HER2*/neu-amplified breast cancers

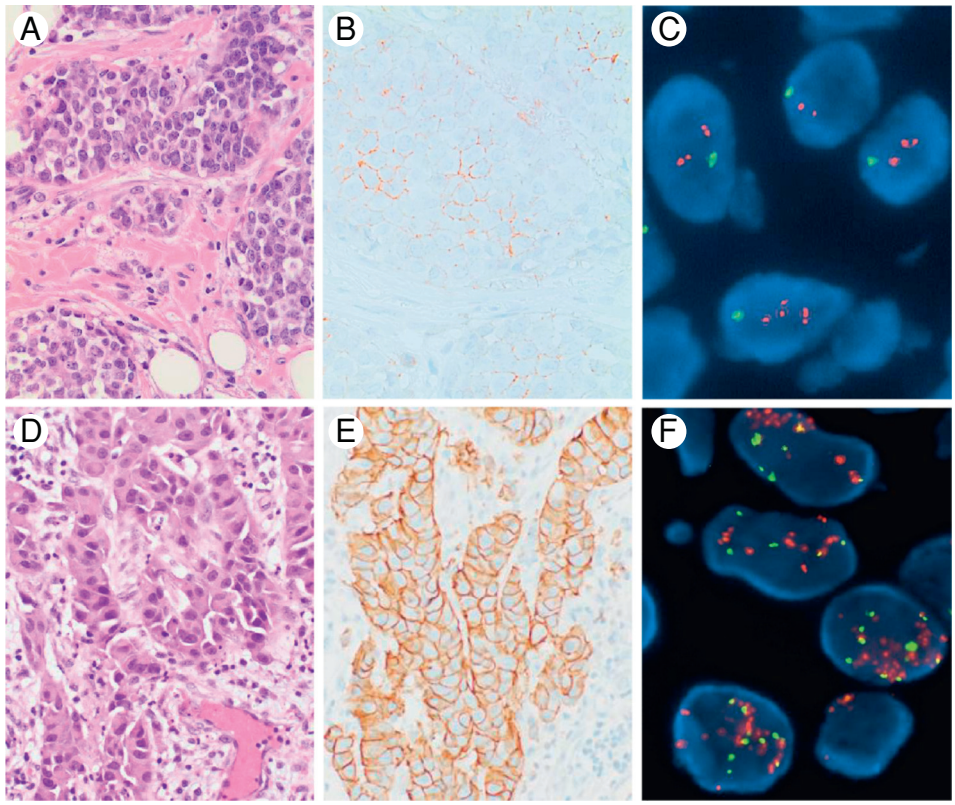

Figure Example of a tumor with *HER2* signals/nucleus less than 4 and *HER2*/*CEP17* ratio of 2 or greater (top row) and a *HER2*-amplified tumor with *HER2* signals/nucleus of 4 or greater (bottom row). A, H&E-stained section of IDC (original magnification ×40). B, *HER2* IHC showing negative staining (1+; ×40). C, FISH for *HER2* (Spectrum Orange) and *CEP17* (Spectrum Green) with *HER2/CEP17* ratio of 2.7 and average *HER2* copy number of 3.3. D, H&E-stained section of IDC of breast (×40). E, HER 2 IHC showing positive staining (3+; ×40). F, FISH for *HER2* (Spectrum Orange) and *CEP17* (Spectrum Green) with *HER2/CEP17* ratio of 2.7 and average *HER2* copy number of greater than 20.

Table 2 Comparison of *HER2* IHC score in 2 groups of tumors with amplified *HER2* FISH results

HER2 IHC results	*HER2* FISH results	
	Group A (*HER2*/*CEP17* ≥2; *HER2* copy <4)	Group B (*HER2* amplified; *HER2* copy ≥4)
HER2 negative (0, 1+)	11 (61.1%)	14 (9.3%)
HER2 equivocal (2+)	7 (38.9%)	36 (23.8%)
HER2 positive (3+)	0	101 (66.9%)
Total	18	151

with a *HER2*/neu copy number of 4 or greater (group B). Various tumor characteristics were compared between these groups using Fisher exact test and 2-tailed *t* test. For all statistical tests, a *P* value less than .05 was considered significant.

3. Results

Dual-probe FISH analysis to determine *HER2* gene status was performed on 1201 breast cancers during the study period. The cancers were from 1072 women and 4 men (mean age, 58 years [age range, 22-99 years]). Of the 1201 cases, 169 (14%) were *HER2* amplified, 910 (76%) were non amplified, and 122 (10%) showed equivocal results by ASCO/CAP 2013 guidelines. Of the *HER2*-amplified cases, 151 (89.3%) had *HER2* copy number of 4.0 or greater (group B) and 18 (10.7%) demonstrated mean *HER2* signals/cell of less than 4 and *HER2*/*CEP17* ratio of 2 or greater (group A). Upon review of the 18 group A cases, the gene copy numbers and gene/control ratios for the 2 technologists that independently reviewed these cases were not significantly different. Group A consisted of 18 women with mean age of 54.4 years (age range, 33-79 years). Clinicopathological characteristics of the tumors are summarized in Table 1. Representative images of group A and B cases are shown in Figure.

Table 3 Clinicopathological characteristic of 2 groups of tumors with amplified *HER2* FISH results

Pathologic characteristics	*HER2* FISH results	
	Group A (*HER2*/*CEP17* ≥2; *HER2* copy <4)	Group B (*HER2* amplified; *HER2* copy ≥4)
Histologic grade		
Low grade	2 (14.2%)	5 (3.7%)
Intermediate grade	6 (42.9%)	64 (47.4%)
High grade	6 (42.9%)	66 (48.9%)
NA	4	16
Tumor size		
≤2 cm	7 (58.3%)	49 (57.6%)
>2 cm	5 (41.7%)	36 (42.4%)
NA	6	66
ALN status		
ALN+	5 (38.5%)	59 (50.4%)
ALN−	8 (61.5%)	58 (49.6%)
NA	5	34
Tumors types		
IDC	11 (73.3%)	117 (84.8%)
Non-IDC	4 (26.7%)	21 (15.2%)
NA	3	13
ER status		
ER+	13 (72.2%)	109 (72.2%)
ER−	5 (27.8%)	42 (27.8%)
PR status		
PR+	14 (77.8%)	87 (57.6%)
PR−	4 (22.2%)	64 (42.4%)
HER2 IHC		
Negative/equivocal	18 (100%)	50 (33.1%)
Positive	0	101 (66.9%)
Total	18	151
Age (y), mean (range)	54.4 (33-79)	54.9 (24-89)

Abbreviations: ALN, axillary lymph nodes; NA, not available.

3.1. Distribution of *HER2* IHC results

HER2 IHC results were available for all the cases (Table 1). The 18 group A cases were associated with low *HER2* protein expression: 11 (61.1%) showed negative *HER2* IHC (0 and 1+), 7 (38.9%) were equivocal (2+), and none was reported as positive (3+). In comparison, most *HER2*-amplified cases with at least 4 *HER2* copies/cell demonstrated *HER2* protein overexpression, 101 (66.9%) were positive by HER IHC, 14 (9.3%) were negative, and 36 (23.8%) were equivocal ($P < .01$; Table 2). None of the group A scores changed on review, and the distribution of staining was not notably heterogeneous.

3.2. Clinicopathological characteristics

Clinicopathological parameters of group A tumors (*HER2/CEP17* ratio ≥2 and mean *HER2* signals/cell <4) and group B tumors (*HER2* amplified with *HER2* copy ≥4.0) are summarized in Table 3. Hormone receptor status was known for all the cases. Most of the 18 tumors from group A expressed estrogen receptor (ER; 72.2%) and progesterone receptor (PR; 77.8%). In comparison, 72.2% of 151 tumors from group B expressed ER, and only 57.6% were PR positive.

Among the cases from group A with available clinicopathological data, most were invasive ductal carcinoma (IDC; 73.3%), grade 1 to 2 cancers (57.1%), with tumor size of 2 cm or less (58.3%), and no axillary lymph node involvement (61.5%). The cases from group B were also mostly IDC (84.8%), with tumor size of 2 cm or less (57.6%), high-grade tumors (48.9%) and showed higher percentage of axillary lymph node involvement (50.4%). Histologic grade 1 tumors constituted 14.2% of group A cases and only 3.7% of group B tumors. These numerical differences notwithstanding, no statistically significant differences were demonstrable regarding the aforementioned clinicopathological features between group A and B cases.

4. Discussion

It is not entirely clear that breast cancers that are classified as *HER2* positive with *HER2*/*CEP17* ratio 2 or greater and *HER2* signals/cell less than 4 are truly amplified in a biologically relevant manner, or whether this finding is merely an artifact of testing methodology and interpretation. This *HER2* FISH profile frequently occurs in the setting of low mean *HER2* copy number (<4) and loss of chromosome 17 copy number due to true monosomy (loss of a chromosome) or loss of a portion of the chromosome 17. Few studies have evaluated the clinical impact of monosomy 17 in *HER2* testing, and definitions have varied in the studies [9,19,20]. Some authors have noted the somewhat counterintuitive nature of the scenario wherein a gene is considered amplified, although there are fewer copies of the gene than would be present in the normal cell in the S phase [12]. The 2013 ASCO/CAP recommendation that cases such as those in our group A be classified as *HER2* amplified is by and large based on findings from the large-scale HERceptin Adjuvant trial, which included a subgroup of 48 patients with an average of less than 4 *HER2* copies per cell and a ratio of at least 2, and the finding that there was no overt trend toward nonresponsiveness to *HER2*-directed therapy in this subset of patients [11]. However, the authors of the 2013 ASCO/CAP recommendation also noted that "several members of the Update committee expressed concern about describing" cases meeting group A criteria as *HER2* amplified [5]. The authors also subsequently wrote that repeat testing using the same or another specimen is wholly appropriate in this setting and that one motivation behind the recommendation was the absence of conclusive evidence that would have specifically justified denying group A patients *HER2*-directed therapy [21]. Page et al [9], in a more recent study that defined *HER2*-amplified cancers with chromosome 17 monosomy as CEP17 signal of less than 1.5 per nucleus and a *HER2/CEP17* ratio of 2.0 or greater, showed that such trastuzumab-treated patients have similar outcomes to other *HER2* positive patients. However, whether so-defined *HER2*-monosomy tumors were closer in clinicopathological profile to *HER2*-nonamplified tumors was not specifically addressed and that study did not specifically evaluate the cases with a *HER2* copy number less than 4 [9]. Press et al [14] examined the issue more directly. The authors defined 5 groups of *HER2* FISH results and assessed the group-specific responses to *HER2*-targeted therapy. The authors reported no apparent benefit from trastuzumab therapy in their "group 2" cancers, which comprised cases with mean *HER2* signals/cell of less than 4 and *HER2*/*CEP17* ratio of 2 or greater [14]. In the N9831 trastuzumab trial, cases with a *HER2*/*CEP17* ratio of greater 2.0 that were negative by IHC did not receive definite benefit from treatment with trastuzumab [22]. Based in part on the aforementioned studies, in the 2018 ASCO/CAP guidelines, it was recommended that cases in our group A with a negative IHC result be classified as *HER2* negative, with the following comment: "evidence is limited on the efficacy of *HER2*-targeted therapy in the small subset of cases with an *HER2*/*CEP17* ratio of 2.0 and an average *HER2* copy number of <4.0 per cell. In the first generation of adjuvant trastuzumab trials, patients in this subgroup who were randomly assigned to the trastuzumab arm did not seem to derive an improvement in disease-free or overall survival, but there were too few such cases to draw definitive conclusions" [16].

In the current study, we examined the frequency of the breast cancers with the aforementioned dual-probe FISH profile (mean *HER2* signals/cell <4 and *HER2*/*CEP17* ratio ≥2), described their clinicopathological profile, and compared this profile with those of *HER2*-amplified cancers with a *HER2* copy number of 4 or greater to determine whether significant differences are discernible between these 2 subgroups that, according to current 2013 ASCO/CAP criteria, are routinely classified as *HER2* amplified. Because our institutional practice is to perform IHC and FISH on every case, we also

assessed how our group A cases correlated with immunohistochemical results, as compared with classically defined *HER2*-amplified cases.

We found that cases with mean *HER2* signals/cell less than 4 and *HER2*/*CEP17* ratio of 2 of greater accounted for 10.7% of all *HER2*-amplified cases and 1.5% of all breast cancers classified by 2013 ASCO/CAP criteria. The latter figure (1.5%) is on the upper end of the 0.4% to 1.4% range of frequencies reported in prior studies [10,13-15,23]. Among our *HER2*-amplified breast cancers with less than 4 *HER2* copy/cell, 61.1% were negative by *HER2* IHC and none of these tumors were positive (score 3+). In contrast, most of the *HER2*-amplified tumors with at least 4 *HER2* copy numbers showed 3+ *HER2* protein overexpression ($P < .01$). Similarly, Ballard et al [10] reported that most of *HER2*-amplified cases in monosomy category (*HER2* signals/cell <4 and *HER2*/*CEP17* ratio ≥2) showed negative and equivocal *HER2* IHC results.

In our study, most tumors with mean *HER2* signals/cell less than 4 and *HER2*/*CEP17* ratio of 2 or greater were hormone receptor positive and grade 1 to 2 cancers (57.1%), and showed no axillary lymph node involvement (61.5%). Ballard et al [10] also observed a relatively higher percentage of grade 1 cancers (13.3%) in their monosomy group, similar to our findings (14.4%). There was a suggestion in our data that group B tumors show a higher frequency of high-grade tumors and node involvement. However, perhaps because of the small sample for group A, we could not demonstrate statistically significant differences between our 2 groups regarding clinicopathological parameters. More generally, our study is limited by its retrospective nature and the inability, due to the rarity of the central result, of correlating findings with treatment and outcome data. Our results should be interpreted within the context of these limitations.

In summary, tumors with *HER2*/*CEP17* ratio of 2 or greater and *HER2* copy number less than 4 represent 1.5% of all breast cancers and 10.7% of all *HER2*-amplified breast cancers classified as such based on 2013 ASCO/CAP criteria. These cases are associated with low or absent *HER2* protein expression: most [61%] showed IHC scores of 1+ or 0, which significantly distinguished them from cases with *HER2*/*CEP17* ratio of 2 or greater and *HER2* copy number of 4 or greater, most (66.9%) of which demonstrated 3+ scores by IHC. The high positive correlation rate between *HER2* status as determined IHC and FISH in our group B is typical of historically reported cohorts of breast cancers [5,13,19]. The notable discordance between *HER2* status as determined by IHC and FISH in our group A suggests that there are inherent biologic differences between our 2 groups. This finding, in combination with prior reports that group A patients derive no significant benefit from trastuzumab therapy [14], bolsters the 2018 ASCO/CAP guideline that such cases not be classified as *HER2* amplified without further workup. By 2018 ASCO/CAP criteria, with its attendant requisite immunohistochemical correlation, most of our group A cases would be classified as *HER2* negative.

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