# Telomere shortening in breast cancer correlates with the pathological features of tumor progression

MAKOTO KAMMORI[1,2,4*], YOSHIYUKI SUGISHITA[2*], TAKAHIRO OKAMOTO[4], MAKIO KOBAYASHI[5], KAZUKO YAMAZAKI[2], EMIKO YAMADA[3] and TETSU YAMADA[1,2]

Departments of [1]Surgery, [2]Laboratory and [3]Medicine, Kanaji Thyroid Hospital, Kita-ku, Tokyo 114-0015; [4]Department of Surgery, Division of Surgical Endocrinology and [5]Department of Pathology 1, Tokyo Women's Medical University, Shinjuku-ku, Tokyo 162-0054, Japan

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**Abstract.** Telomeres are involved in the maintenance of genomic stability. Telomere alteration has been observed in most human cancer types, and is known to be a feature of malignancy. The aim of the present study was to evaluate whether the telomere length of breast cancer cells correlates with TNM stage and several pathological features. We investigated a total of 44 breast cancers, including 17 scirrhous, 15 papillotubular and 12 solid-tubular carcinomas. Telomere lengths were determined by tissue quantitative fluorescence *in situ* hybridization (Q-FISH), and compared according to the TNM stage, histological tumor size, lymph node metastases, vascular invasion and immunohistochemical status (ER, PR, HER2 status and Ki67 labeling index). In all histological types, telomeres of cancer cells were significantly shorter than those of normal epithelial cells. Mean telomere length was significantly less in patients with TNM stage III, and in those with large tumors, lymph node metastases and vascular invasion. Our results suggest that the telomere length of cancer cells is strongly correlated with the degree of cancer progression.

## Introduction

Telomeres are protective structures that cap the ends of eukaryotic chromosomes (1), comprising multiple 5'-TTAGGG-3' repeats and ending in a single-stranded overhang of the G-rich sequence (2). Telomeres protect chromosome ends from end-to-end fusion, nucleolytic decay, degradation and atypical recombination (3). The telomere repeat sequence becomes shortened by each cell division, DNA damage due to oxidative stress or through changes in telomere-associated proteins (4,5). It has been proposed that telomere shortening is an important biological factor involved in carcinogenesis, cell senescence, cell replication, cell immortality and aging (6-8). Accumulating evidence has led to the hypothesis that telomere dysfunction contributes to genetic changes intrinsic to the development and progression of tumors (9-11), and that telomere length can be considered as a biological index of malignant potential (12). It has been reported that telomere shortening occurs in breast cancer cells (13) and contributes to tumor progression in numerous cancer types including breast cancer (14,15).

Breast cancer is the most frequent malignant disease and the leading cause of cancer-related death among women worldwide. Globally, 1.4 million new cases of breast cancer are diagnosed annually of which approximately one third are fatal (16). It is well established that telomere shortening is present in the majority of *in situ* and invasive carcinomas (14) including breast cancers (17,18). In addition to their role in tumor initiation, short dysfunctional telomeres affect disease progression. Previous studies have shown that telomeres are shorter in grade III tumors (19), that telomere shortening is correlated with aneuploidy and lymph node metastasis (20), and that shorter telomeres are associated with higher stage and histological grade (21). Moreover, it has been reported that short telomeres are associated with tumor size, nodal involvement and TNM stage (22). However, another study found no correlation between telomere length and tumor volume, grading, node status or expression of estrogen receptor (ER) and progesterone receptor (PR) (23).

The aim of the present study was to clarify whether the telomeres in three histological types of breast cancer (scirrhous, papillotubular and solid-tubular carcinomas) are shorter than those of normal epithelial cells, and whether telomere length is correlated with TNM stage and several pathologic factors. For this purpose, we measured telomere lengths in breast cancer using quantitative fluorescence *in situ* hybridization (Q-FISH), which allowed us to estimate the telomere lengths of individual cells in each section.

## Materials and methods

*Tissue specimens*. We examined a total of 44 breast cancers, including 17 scirrhous, 15 papillotubular and 12 solid-tubular

*Correspondence to:* Dr Makoto Kammori, Department of Surgery, Kanaji Thyroid Hospital, 1-5-6 Nakazato, Kita-ku, Tokyo 114-0015, Japan
E-mail: kammori@kanaji.jp

*Contributed equally

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carcinomas. Both tumor and adjacent normal tissues were obtained from each individual patient and embedded in paraffin using standard processing procedures. Sections 4-$\mu$m thick were prepared for tissue Q-FISH and immunohistochemistry (IHC) analysis. All samples were collected at the Division of Surgical Endocrinology of Tokyo Women's Medical University, Tokyo, Japan, after obtaining informed consent from all of the patients. Table I summarizes the clinicopathological results. All pathologic examinations were conducted by one of the authors who was a trained pathologist (M.K.).

*Tissue Q-FISH*. Tissue Q-FISH was performed as previously described (24-26). In brief, tissue sections were deparaffinized and treated with 0.2 N HCl and 1 M sodium thiocyanate at 80˚C, 1% pepsin at 37˚C and 10 mg/ml RNase at 37˚C. A peptide nucleic acid (PNA) telomere probe conjugated to Cy3 (telo C Cy3 probe, 5'-CCCTAACCCTAACCCTAA-3'); and a PNA centromere probe conjugated to fluorescein isothiocyanate (FITC) (Cenp 1 probe, 5'-CTTCGTTGGAAACGGGGT-3') (both from Fasmac, Kanagawa, Japan) were applied to each section. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St, Louis, MO, USA).

FISH images were captured by a CCD camera attached to an epifluorescence microscope (Eclipse 90i; Nikon, Tokyo, Japan) equipped with a triple band-pass filter set for DAPI/FITC/Cy3 (61000v2m; Chroma Technology Corp., Rockingham, VT, USA) and a x40 objective lens (Plan Fluor x40/0.75; Nikon). Microscope control and image recording were performed using Image-Pro Plus software (version 6.3; Media Cybernetics, Bethesda, MD, USA). The recorded images were analyzed as previously described using original software prepared by our colleague (S.P.) (24-26), 'Tissue Telo Version 3.2', which allows manual identification of nuclear regions from the composite color image: DAPI (blue channel), FITC (green) and Cy3 (red). Fluorescence intensities of telomere (Cy3) and centromere signals (FITC) for each nucleus were measured, and then the telomere-centromere ratio (TCR) was calculated, since there is no guarantee that all information on telomere signals will be acquired within any given tissue section.

*IHC*. The expression of ER, PR, human epidermal growth factor receptor 2 (HER2) and Ki67 (Dako Japan, Tokyo, Japan) protein was determined by IHC staining. For ER, PR and Ki67, the tissue sections were pretreated with Tris-EDTA buffer solution (pH 9.0) at 95˚C, and for HER2 with citrate buffer solution (pH 6.0) at 95˚C. After incubation with the primary antibody for 2 h, visualization was performed using a polymer IHC detection system (EnVision kit; Dako Japan). The Ki67 labeling index (LI) was calculated by counting at least 500 cells in each specimen.

*Statistical analysis*. The significance of differences in mean TCR was examined by Welch's t-test. When three groups were compared, we used one-way ANOVA and the Tukey-Kramer post hoc test. For analyzing correlations between continuous variables (pathological tumor size and Ki67 LI) and TCR, Pearson's correlation coefficient was calculated. Differences at $P<0.05$ were considered to indicate a statistically significant result.

Table I. Clinicopathological characteristics of the study patients.

| Histological type | Scirrhous carcinoma (n=17) | Papillotubular carcinoma (n=15) | Solid-tubular carcinoma (n=12) |
|---|---|---|---|
| Clinical characteristics | | | |
| Age (years)[a] | 55.5±11.0 | 52.0±17.0 | 56.1±14.3 |
| TNM stage | | | |
| 0 (DCIS) | 1 | 1 | 0 |
| I | 5 | 5 | 6 |
| II | 9 | 8 | 5 |
| III | 2 | 1 | 1 |
| Pathological characteristics | | | |
| Tumor size (mm)[a] | 22.2±12.0 | 23.3±15.9 | 21.4±12.1 |
| Lymph node metastases[b] | | | |
| pN0 | 9 | 13 | 9 |
| pN1 | 3 | 1 | 3 |
| pN3 | 2 | 0 | 0 |
| Vascular invasion[b] | | | |
| + | 10 | 8 | 7 |
| - | 6 | 7 | 4 |
| ER status[b] | | | |
| + | 14 | 11 | 6 |
| - | 1 | 3 | 6 |
| PR status[b] | | | |
| + | 14 | 9 | 5 |
| - | 1 | 5 | 7 |
| HER2 status[b] | | | |
| 0-1 | 11 | 12 | 8 |
| 2 | 3 | 2 | 2 |
| 3 | 1 | 1 | 2 |
| Ki67 LI (%)[a] | 6.8±11.6 | 13.0±14.4 | 11.4±9.7 |

[a]Data are expressed as mean ± standard deviation. [b]Including unknown cases. LI, labeling index.

## Results

*Telomere length (TCR distribution) in cancer and adjacent normal breast tissues*. Telomere signals were evident within nuclei as small red spots in normal epithelial cells (Fig. 1a-1). Telomere signals of tumor cells in scirrhous carcinomas (Fig. 1b-1 and b-2) were weaker than those in adjacent n ormal epithelial cells (Fig. 1a-1 and a-2). Cancer cells most frequently had very short telomeres, yet a few cells had long telomeres such as TCR >4, and the peak frequency of TCR was in <1 (Fig. 2a-2, b-2 and c-2). On the other hand, normal epithelial cells had relatively long telomeres (TCR >4) and the TCR had a very wide distribution (Fig. 2a-1, b-1 and c-1). The

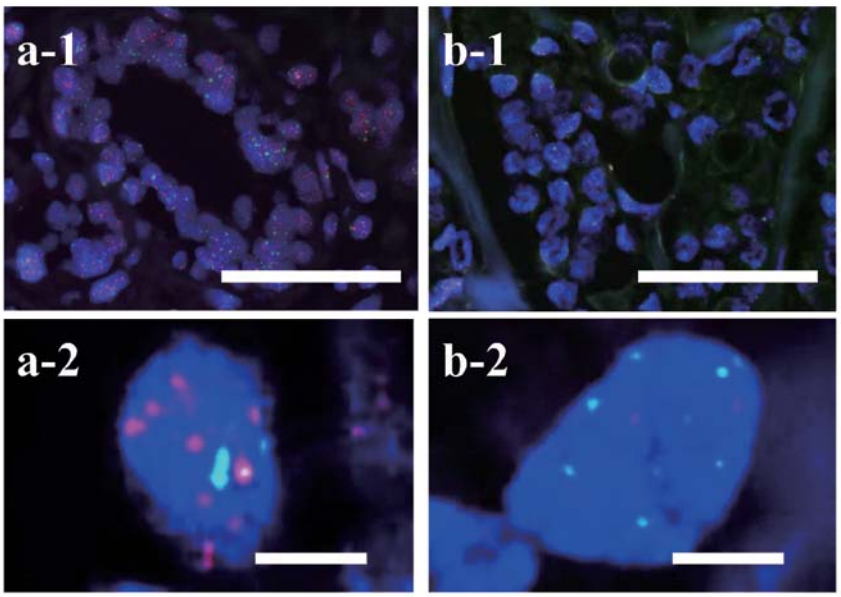


Figure 1. Tissue Q-FISH images of normal epithelial cells and scirrhous carcinomas. (a-1 and a-2) Normal epithelial cells; (b-1 and b-2) scirrhous carcinomas. The nuclei are stained with blue fluorescence (DAPI). Red (Cy3) and green dots (FITC) within nuclei indicate telomere and centromere signals, respectively. Original magnification, x40. The scale bar is 50 and 5 $\mu$m in a-1, b-1 and a-2, b-2, respectively. Q-FISH, quantitative fluorescence *in situ* hybridization; DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate.

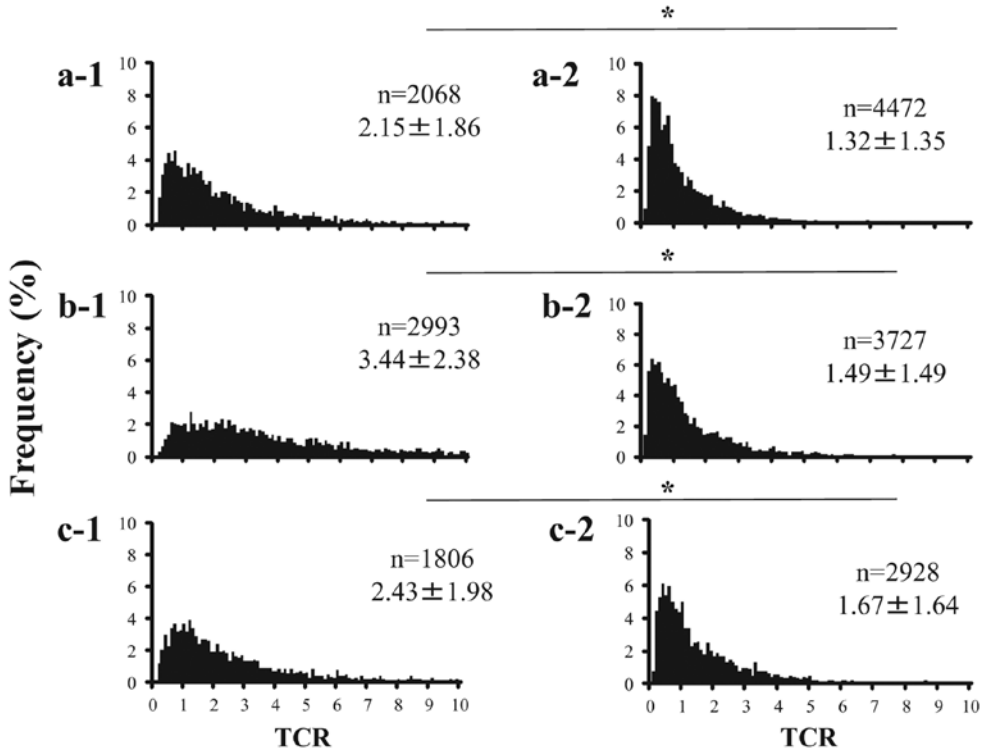


Figure 2. Histograms of TCR distribution in various cell types. a, Scirrhous carcinomas and adjacent normal cells; b, papillotubular carcinomas and adjacent normal cells; c, solid-tubular carcinomas and adjacent normal cells; 1, normal epithelial and 2, tumor cells. Number of analyzed nuclei (n) and mean TCR ± standard deviation are indicated in each histogram. Asterisks indicate a significant difference determined using Welch's t-test (*P<0.05). TCR, telomere-centromere ratio.

mean TCR of cancer cells was significantly lower than that of normal epithelial cells in all histological types (Fig. 2, P<0.05).

*Relationship between telomere length and several pathological and immunohistochemical factors*. In the present study, associations between mean TCR and clinical TNM stage, histological tumor size, pathologically proven lymph node metastasis, vascular invasion, ER, PR, HER2 status and Ki67 LI were analyzed (Fig. 3). Tumor size was divided into three groups (≤20, 20-50 and >50 mm) in accordance with

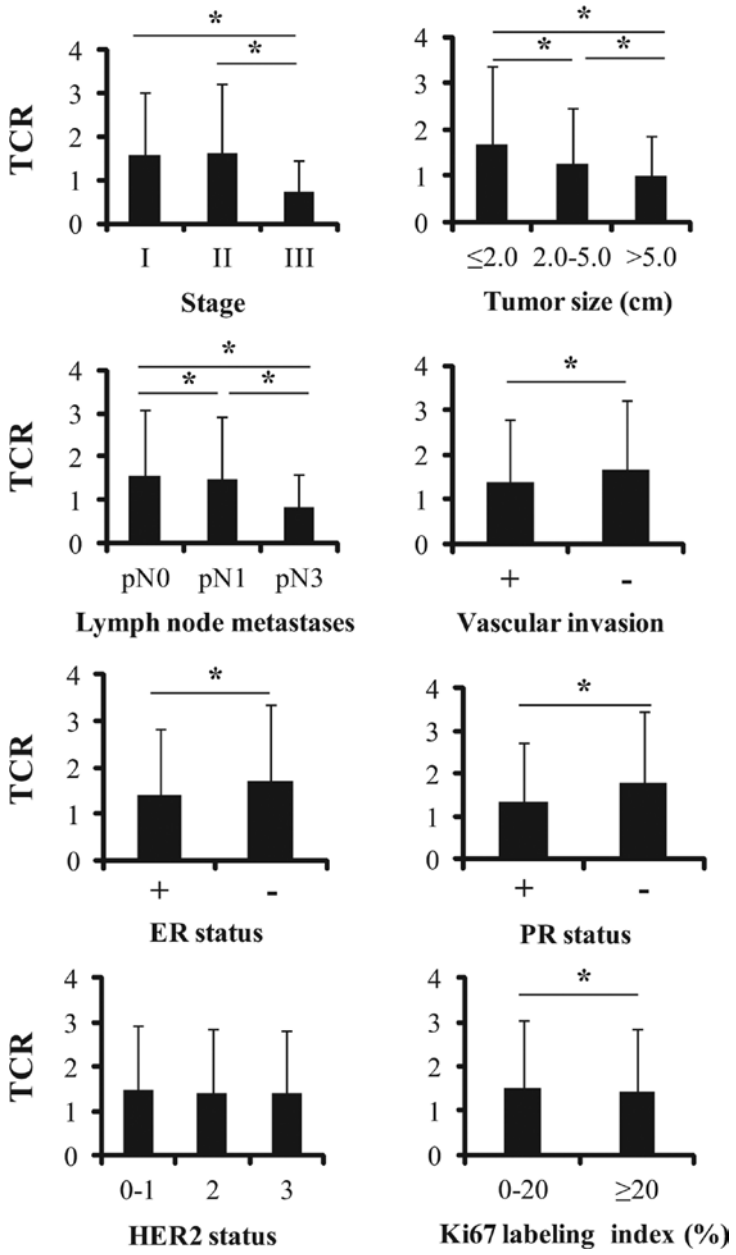


Figure 3. Comparison of mean TCR according to several pathological factors. Error bars indicate standard deviation. Asterisks indicate a significant difference determined using Welch's t-test (*P<0.05). Three groups were compared using one-way ANOVA and the Tukey-Kramer post hoc test (*P<0.05). TCR, telomere-centromere ratio.

the UICC criteria. For Ki67 LI, though cut-off values have varied among previous studies (27-30), a nuclear LI of ≥20% was considered high and one of <20% was considered low for the purposes of the present study. Telomere shortening was associated with TNM stage III, a large tumor size, presence of a large number of lymph node metastases, presence of vascular invasion, ER positivity and PR positivity (Fig. 3). However, HER2 status did not correlate with telomere length (Fig. 3). Telomeres lengths of Ki67 LI ≥20% patients were shorter than those of LI <20% patients (Fig. 3). However, there was no significant correlation between Ki67 LI and mean TCR when we analyzed the Pearson's correlation coefficient (P=0.806, Fig. 4).

We analyzed the correlation between histological tumor size and mean TCR by calculation of Pearson's correlation coefficient. Although correlation coefficient was not so strong ($R^2$=0.102), statistically significant inverse correlation was observed (P=0.037, Fig. 4).

## Discussion

Generally, the cells of malignant tumors including breast cancer have shorter telomeres than the corresponding cells in normal tissue (13,21), and telomere dysfunction or shortening has been considered as a negative prognostic indicator in patients with solid tumors (31,32), including breast cancer (33,34). Furthermore, it has been reported that telomere shortening contributes to tumor progression (14,15). In the present study, using tissue Q-FISH technique, we evaluated telomere length in breast cancer tissues and also

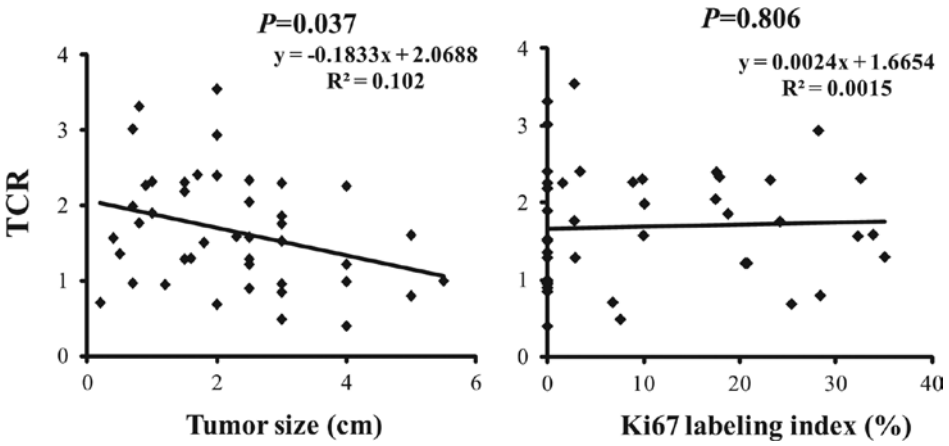


Figure 4. Association of mean TCR with tumor size and Ki67 LI. There is an inverse relationship between mean TCR and tumor size, yet no relationship between mean TCR and Ki67 LI. TCR, telomere-centromere ratio; LI, labeling index.

confirmed that cancer cells had shorter telomeres than the adjacent normal epithelial cells in three histological types of carcinoma (scirrhous, papillotubular and solid-tubular carcinomas). Tissue Q-FISH effectively estimates telomere length in different cell types using separate PNA probes for telomeres and centromeres, thus allowing specific evaluation for cancer cells. We obtained the telomere-centromere ratio (TCR) as a parameter representative of telomere length, and many previous studies have verified its accuracy for this purpose (24-26). Moreover, we evaluated whether telomere length was correlated with several pathological features, indicating tumor progression.

The principal conclusion emerging from the present study was that telomere shortening is associated with some parameters of cancer progression. Mean telomere length was significantly less in patients with TNM stage III disease, a large tumor size, a large number of lymph node metastases and vascular invasion. As previously described, several studies of breast cancer have revealed that telomere length is associated with TNM stage, tumor size, nodal involvement and prognosis (21,22,35). On the other hand, some studies have found no correlation between telomere length and tumor volume, grading, nodal or ER and PR status (20,23). Our present findings are in agreement with the results of some of these studies (21,22,35), and suggest that telomere length may be a useful index of tumor aggressiveness in breast cancer. Although tumor size has been the traditional prognostic factor, it has been regarded as one of the most powerful predictors of tumor behavior in breast cancer (36-38). Since the present study demonstrated an inverse relationship between mean TCR and histologically evident tumor size, our results reinforce the assumption that telomere length reflects the prognosis.

Recently, breast cancer has been classified into different molecular subtypes with different biological features, clinical outcome and response to therapy (39-42). These subtypes can be distinguished on the basis of ER, PR and HER2 status: luminal A ($ER^+$ and/or $PR^+$, $HER2^-$), luminal B ($ER^+$ and/or $PR^+$, $HER2^+$), HER2 ($ER^-$ and $PR^-$, $HER2^+$) and basal-like ($ER^-$, $PR^-$, $HER2^-$). Various studies have indicated the importance of using the proliferation index (Ki67 LI) to distinguish between the luminal A and B subtypes (27,43). In addition, telomere shortening is reportedly associated with specific breast cancer subtypes; some studies have indicated that ER- and/or PR-negative cancers have shorter telomeres than ER- and/or PR-positive cases (44,45). In the present study, both ER- and PR-positive tumors had shorter telomeres than ER- and PR-negative tumors, respectively, being contradictory to results obtained in previous studies. This may have been due to the fact that both the ER- and PR-positive groups in the present study included pN3 cases with very short telomeres. However, due to the limited number of patients with ER- and/or PR-negative tumors, we considered that these results should be viewed with caution, and that no clear conclusion can yet to be drawn. Furthermore, telomere length did not differ significantly according to the HER2 status, and no correlation between Ki67 LI and telomere length was evident. Accordingly, the data obtained in the present study were considered insufficient for discussing their relationship with molecular subtypes such as the luminal classification. However, as previously described, it is thought that telomere metabolism is a very important factor impacting on tumor behavior, and that tissue Q-FISH provides an accurate estimation of telomere length. We considered that further studies will be needed to evaluate telomere length using the tissue Q-FISH method in relation to luminal molecular subtypes.

In summary, we have demonstrated a significant correlation between telomere length and TNM stage, tumor size and lymph node metastasis in breast cancer. Our present results suggest that the telomere length of cancer cells is strongly correlated with cancer progression.

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