Overexpression of Androgen Receptor and Forkhead-box A1 Protein in Apocrine Breast Carcinoma

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Abstract. *Aim: Apocrine breast carcinoma often lacks estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor type-2 (HER2) expression. Accordingly, development of a new treatment strategy is important for this type of cancer. The growth stimulus through the androgen receptor (AR) can be a candidate for targeted treatment. Therefore, we examined the factors related to AR transcription. Materials and Methods: We immunohistochemically evaluated 54 apocrine cancer lesions for ER, PgR, AR, HER2, Ki-67, forkhead-box protein A1 (FOXA1), and prostate-specific antigen (PSA) expression. Results: ER, PgR, and HER2 were expressed at a low level, thus 44 out of 54 (81.4%) cases were of triple-negative breast cancer. AR, PSA and FOXA1 were expressed in 100% (54/54), 48% (26/54) and 93% (50/54) of cases, respectively. Conclusion: Most of apocrine breast carcinomas were immunohistochemically-positive for AR and FOXA1. Anti-androgenic therapies can potentially serve as a cancer-targeting therapy for apocrine breast carcinoma.*

Apocrine breast carcinoma is histologically-defined as a unique type of invasive ductal carcinoma of the breast (1). Apocrine cancer cells have large, round nuclei and abundant, eosinophilic, granular, and sharp-bordered cytoplasm. Apocrine carcinoma is a rare variant of breast carcinoma and accounts for approximately 0.3-4% of cases (2, 3).

The immunohistochemical staining pattern for this type of breast cancer is characterized by androgen receptor (AR) positivity and, often, estrogen receptor (ER) and progesterone receptor (PgR) negativity. Human epidermal growth factor receptor type-2 (HER2) is expressed, frequently at a low level, in apocrine carcinoma, and therefore, HER2-negative apocrine carcinoma can be phenotyped as triple-negative breast cancer (TNBC).

Compared to common breast carcinoma types, varying results for the characterization and prognosis of apocrine carcinoma have been reported (3-7). Generally, TNBC is characterized by high-grade nuclear atypia and aggressive clinical behavior with a poor prognosis (6). Because TNBCs are resistant to endocrine and anti-HER2 therapy, they are generally managed using standard treatment with non-specific cytotoxic agents. Thus, development of a new treatment strategy for this type of breast cancer is an important issue.

Celis *et al*. reported that apocrine metaplasia in benign breast lesions and classical apocrine carcinoma express the $ER^-/PgR^-/AR^+$ phenotype (5). Tsutsumi immunohistochemically defined apocrine-type invasive ductal carcinoma as having an $ER^-/PgR^-/AR^+$ phenotype, to differentiate it from basal-like TNBC (8). In another *in vitro* study, molecular apocrine tumors were recognized as an additional sub-category of breast cancer, characterized by an ER^-/AR^+ phenotype on the basis of their gene expression signatur (9). Molecular apocrine tumors have an expression profile similar to that of ER^+ luminal breast cancer. Using a cell line model of ER^-/AR^+ molecular apocrine tumors, Robinson *et al*. mapped global AR binding in a near-perfect sub-class of forkhead-box protein A1 (FOXA1)-binding regions (10). FOXA1 is an intracellular transcription factor and is essential for ER-mediated initiation of transcription (11-13). FOXA1 is usually highly expressed in ER-positive breast carcinomas (12, 14, 15). Moreover, FOXA1 participates not only in the transcription by ER but also in the transcription by AR. Therefore, it was hypothesized that AR functionality is dependent on FOXA1.

AR and FOXA1 are expected to be the main target molecules for the effective treatment of apocrine cancer. In the present study, apocrine carcinoma was morphologically-

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defined on the basis of histological criteria. A total of 54 apocrine breast cancer lesions were evaluated immunohistochemically for ER, PgR, AR, HER2, Ki-67, FOXA1, and prostate-specific antigen (PSA) expression. Appropriate pathological evaluation of classical apocrine carcinoma, which may be distinctive from basal-like TNBC, may contribute to the development of an effective treatment strategy for apocrine carcinoma.

Materials and Methods

Tumor specimens. Using immunohistochemistry (IHC), ER, PgR, HER2, AR, PSA, and FOXA1 expression was measured in primary breast tumors, and their association with clinicopathological parameters was evaluated. Women with a confirmed histological diagnosis of apocrine breast cancer who underwent radical surgery at the National Hospital Organization Tokyo Medical Center between 2000 and 2012 were retrospectively enrolled in this study.

The histological criteria for the diagnosis of apocrine breast carcinoma included apocrine metaplasia of the cytoplasm and round, centrally, or eccentrically located malignant nuclei containing prominent nucleoli in almost all tumor cells.

Immunohistochemistry. Representative tumor blocks were formalin-fixed, paraffin-embedded, and sectioned at 4-μm thickness. IHC was performed using the streptavidin-biotin method. Antigen retrieval was performed before the application of antibodies. Endogenous peroxidase was blocked using 3% hydrogen peroxide in deionized water.

We used the following clones: mouse monoclonal antibody against ER (clone 1D5; DakoCytomation, Carpinteria, CA, USA); mouse monoclonal antibody against PgR (clone PgR636; Dako); mouse monoclonal antibody against AR (clone AR441; diluted 1:50, Dako); rabbit polyclonal antibody against PSA (clone polyclonal PSA; Dako); mouse monoclonal antibody against HER2 (HercepTest; Dako); goat polyclonal antibody to FOXA1 (clone ab5089; diluted 1:50, Abcam, Cambridge, UK); and mouse monoclonal antibody against Ki-67 (MIB-1; Dako).

The final product of the reaction was visualized using 3,3'-diaminobenzidine, and the nuclei were stained with hematoxylin. A cut-off value of more than 1% stained neoplastic cells was selected for defining positive ER, PgR, and AR immunoreactivity. For the evaluation of PSA expression, cytoplasmic labeling in more than 1% of neoplastic cells was considered positive.

For the determination of HER2 overexpression, we evaluated only the presence and intensity of membranous staining. A score of 2+ was interpreted as weakly-positive, that of 3+ as strongly-positive, and that of 0 or 1+ as negative. Tumors were characterized as HER2-positive if they were scored either as 3+ on IHC or as 2+ on IHC with *HER2* amplification (ratio >2.0) by fluorescence *in situ* hybridization.

For the evaluation of FOXA1 expression, we considered nuclei labeling in more than 1% of neoplastic cells as a positive result. We defined low level staining as an expression of 0-49% and high level staining as an expression of more than 50%. All microscopy slides were independently evaluated by at least two senior pathologists.

Statistical analysis. The associations between FOXA1, PSA expression, and clinicopathological characteristics were assessed using the chi-square test, Fisher's exact test, or the Mann-Whitney *U*-test. The correlation between FOXA1 and PSA expression was examined using Pearson's correlation coefficient. For comparison of the sample means, an independent sample *t*-test was performed. A *p*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS (version 19.0; SPSS Inc., IBM Company, Armonk, NY, USA).

Results

The clinicopathological characteristics of the patients are shown in Table I. All patients were women, and the average age at the time of surgery was 68.4 (range=37-94) years. Almost all patients were post-menopausal, and 24 out of 54 patients (44.4%) were more than 70 years old. Patients with apocrine breast cancer tended to be older than were patients with unselected breast cancer.

ER, PgR, and HER2 were expressed in 5.6% (3/54), 1.9% (1/54), and 13.0% (7/54) of cases, respectively. Most cases, 44 out of 54 (81.4%), were negative for ER, PgR, and HER2 expression, so-called TNBC. AR and PSA were expressed in 100% (54/54) and 48% (26/54) of cases, respectively. FOXA1 was expressed in 93% (50/54) of cases (Table II).

The histological features of apocrine breast carcinoma are shown in Figure 1. Because AR and FOXA1 are nuclear transcription factors, positive staining is identified in the nucleus. In contrast, positive staining for PSA is identified in the cytoplasm.

Correlation of FOXA1 expression with clinicopathological characteristics. The relationship between FOXA1 expression levels and clinicopathological features is presented in Table III. FOXA1 expression did not show a significant association with age, clinical stage, lymph node involvement, or Ki-67 labeling index. FOXA1 positivity was weakly-associated with nuclear grade, but the relationship was not statistically significant.

Correlation of PSA expression with clinicopathological characteristics. The correlation between PSA expression and various clinicopathological features is presented in Table IV. Significant correlations were observed between PSA expression and age, clinical stage, lymph node involvement, and nuclear grade. PSA-negative apocrine cancer cases had a significantly higher nuclear grade than did PSA-positive cases. PSA-negative apocrine cancer cases were often highly infiltrating and had a higher frequency of lymph node metastasis than did PSA-positive cases.

Correlation between FOXA1 and PSA expression. The expression levels of FOXA1 and PSA are plotted in Figure 2. FOXA1 expression was weakly-correlated with PSA expression, but this association was not statistically significant (r=0.239, p=0.082).

Table I. *Clinicopathological characteristics of patients with apocrine breast cancer (n=54).*

Characteristic	Total number (%)
Mean age (years±SD)	68.4±11.0
Stage	
0	15 (27.8)
I	14 (25.9)
II	21 (38.9)
III	4 (7.5)
Menopausal status	
Premenopausal	1 (1.8)
Postmenopausal	53 (98.2)
Number of nodal metastases	
0	43 (79.6)
1-3	9 (16.7)
≥4	2 (3.7)
Nuclear grade	
1	29 (53.7)
2	9 (16.7)
3	16 (29.6)

Table II. *Immunohistochemical staining results and intrinsic subtypes of all cases in this study (n=54).*

Factor	Number (%)
Ki-67 labeling index	
0-14%	45 (83.3)
≥15%	9 (16.7)
AR	
Positive	54 (100)
Negative	0 (0)
FOXA1	
Positive	50 (92.6)
Negative	4 (7.4)
PSA	
Positive	26 (48.1)
Negative	28 (51.9)
ER	
Positive	3 (5.6)
Negative	51 (94.4)
PgR	
Positive	1 (1.9)
Negative	53 (98.1)
HER2	
0	14 (25.9)
1	24 (44.4)
2	10 (18.5)
3	6 (11.1)
Intrinsic subtype	
Luminal A	3 (5.6)
Luminal B	0 (0)
HER2	7 (13.0)
Triple-negative	44 (81.4)

AR: androgen receptor; FOXA1: forkhead-box protein A1; PSA: prostate specific antigen; ER: estrogen receptor; PgR: progesterone receptor; HER2: human epidermal growth factor receptor type 2.

Discussion

In the present study, we only included cases of histologically-defined apocrine breast carcinoma. These cases accounted for 2.9% (54/1898) of all breast cancer cases examined. As assessed by immunostaining, AR was expressed in all cases. Therefore, nuclear staining for AR is considered a feature typical of apocrine breast carcinoma.

A new subtype of breast cancer, molecular apocrine carcinoma, has been designated on the basis of gene expression studies (9). Molecular apocrine tumors are characterized by ER negativity and AR positivity, accounting for 8-14% of all breast cancer cases. In another study, apocrine carcinoma was immunohistochemically-categorized as $ER^-/PgR^-/AR^+$ invasive ductal carcinoma and accounted for 13.1% of all breast cancer cases (8). In combination with our results, we can conclude that apocrine breast cancer is generally AR positive, independent of the definition used. However, molecularly defined apocrine carcinomas account for 8-14% of all breast cancer cases, and their rate is higher than that of morphologically-defined apocrine carcinoma. Many HER2-positive breast carcinomas are included in molecularly-defined apocrine carcinomas (8, 9, 16). The cross-regulation of AR and HER2 has been reported in ER-negative breast cancer by Nadari *et al*. (17). However, there were not many HER2-positive breast carcinomas among the morphologically-defined apocrine cancer cases in our study. Despite the three different definitions for apocrine cancer, AR is believed to play an important role in the carcinogenesis of apocrine cancer.

Similar to ER and PgR, AR is also a steroid hormone nuclear receptor observed in both breast and prostate tissue. In unselected breast cancer cohorts, expression of AR is positively-correlated with ER and PgR expression, as well as with markers of low-grade disease and good differentiation (18). Higher levels of AR expression present a survival advantage, which suggests that AR function may have a tumor-suppressive effect in ER-positive breast cancer cells (19). In contrast, the role of AR expression in ER-negative breast cancer is not fully understood (20). The prognostic impact of AR as an independent predictor of survival in ER-negative disease is less clear, and the results in the literature vary (20, 21). In the molecular apocrine cell line MDA-MB-453, AR is reported to play an oncogenic role by behaving as an ERα surrogate. The oncogenic function of AR in this cell line mimics that of ER in the context of breast cancer and that of AR in the context of prostate cancer. The role of AR in breast epithelial cells may vary from growth inhibition to growth promotion. This role depends on multiple factors, including the

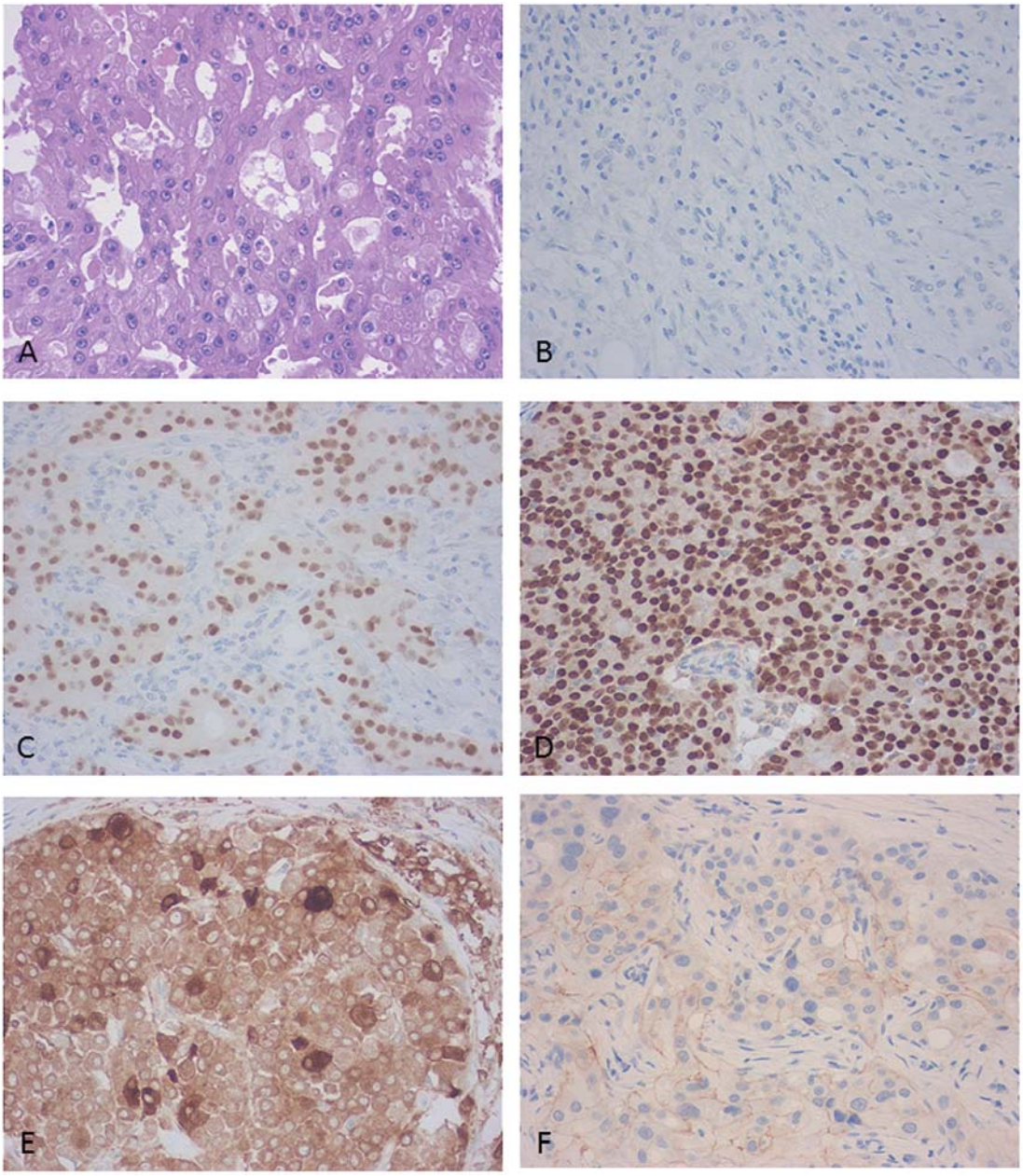

Figure 1. *Immunohistochemical staining for estrogen receptor(ER), androgen receptor(AR), forkhead-box protein A1(FOXA1), prostate specific antigen (PSA), and human epidermal growth factor receptor type-2 (HER2) in apocrine breast cancer. A: Haematoxylin and eosin staining showing apocrine cancer cells with large, round nuclei and abundant, eosinophilic, granular, and sharp-bordered cytoplasm (×200). B: Immunohistochemistry showing negative nuclear staining for ER in a case of apocrine carcinoma (×200). C: Immunohistochemistry showing diffusely positive nuclear staining for AR in a case of apocrine carcinoma (×200). D: Immunohistochemistry showing diffusely positive nuclear staining for FOXA1 in a case of apocrine carcinoma (×200). E: Immunohistochemistry showing diffusely positive cytoplasmic staining for PSA in a case of apocrine carcinoma (×200). F: Immunohistochemistry showing weekly positive membrane staining for HER2 in a case of apocrine carcinoma (×200).*

steroid environment and expression levels of AR, ER, and HER2. It is important to verify the clinical implication of AR expression in patients with apocrine breast cancer.

A number of proteins modulate the activity of ER. These include co-activator and co-repressor molecules as well as the recently described 'pioneer' factor FOXA1. FOXA1 is a main determinant of estrogen–ER action (11). In a previous *in vitro* study, FOXA1 expression was detected in ER-negative breast cancer, and an association with AR expression was described in molecular apocrine tumors

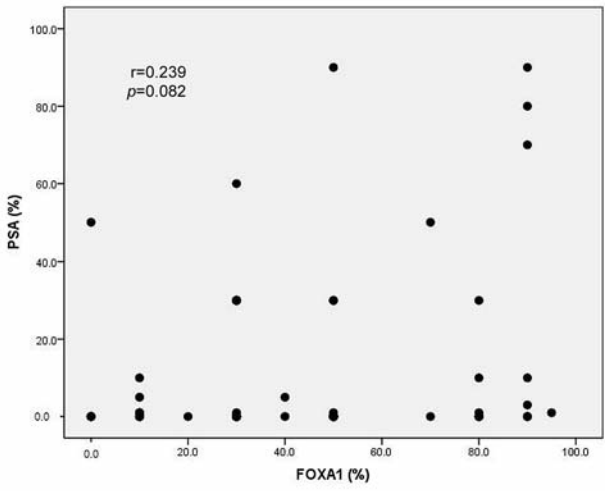

Figure 2. *Correlation between forkhead-box protein A1 (FOXA1) and prostate specific antigen (PSA) expression.*

Table III. *Relationship between forkhead-box protein A1 (FOXA1) expression and clinicopathological features (n=54).*

Factor	FOXA1		*p*-Value
	Low (n=28)	High (n=26)	
Age (years)	68.4±11.3	68.3±11.0	0.99
Stage			
0	6 (21.4)	9 (34.6)	0.27
I	7 (27.3)	7 (26.9)	
II	13 (46.4)	8 (30.1)	
III	2 (7.1)	2 (7.7)	
Invasion			
Intraductal	6 (21.4)	9 (34.6)	0.28
Invasive	22 (78.6)	17 (65.4)	
Lymph node metastasis			
Negative	20 (63.6)	23 (83.9)	0.12
Positive	8 (36.4)	3 (16.1)	
Nuclear grade			
1	11 (36.4)	18 (54.8)	0.060
2	7 (36.4)	2 (16.1)	
3	10 (27.2)	6 (29.1)	
Ki-67 index (mean±SD)	8.1±3.7%	8.2±5.9%	0.93

Table IV. *Relationship between prostate specific antigen (PSA) expression and clinicopathological features (n=54).*

Factor	PSA		*p*-Value
	Negative (n=28)	Positive (n=26)	
Age (years)	71.4±10.7	65.1±10.7	0.034
Stage			
0	3 (10.7)	12 (46.1)	0.001
I	6 (21.4)	8 (30.8)	
II	16 (57.1)	5 (19.2)	
III	3 (10.7)	1 (3.8)	
Invasion			
Intraductal	3 (10.7)	12 (46.1)	0.004
Invasive	25 (89.3)	14 (53.9)	
Lymph node metastasis			
Negative	18 (64.2)	25 (96.2)	0.004
Positive	10 (35.8)	1 (3.8)	
Nuclear grade			
1	10 (36.4)	19 (54.8)	0.006
2	6 (36.4)	3 (16.1)	
3	12 (27.2)	4 (29.1)	
Ki-67 index (mean±SD)	8.3±5.1%	8.1±4.6%	0.89

(10). FOXA1 expression is associated with luminal subtypes and good prognosis in patients with ER-positive tumors (14, 15). Interestingly, FOXA1 expression correlated with low recurrence scores on the Oncotype DX assay. However, expression of FOXA1 in ER-negative primary tumors has been marginally elucidated (1, 11). In the present study, we examined 54 apocrine breast tumor samples and found that most cases (81.4%) were of TNBC, whereas FOXA1 was expressed in 92.6% (50/54) of the cases. In summary, our data show that for apocrine breast carcinomas, the expression of AR and FOXA1 was high, but that of ER was practically negative. It is hypothesized that FOXA1 supports the binding of AR to chromatin to regulate transcriptional activity. Our *in vivo* results are comparable to the findings of *in vitro* studies on molecular apocrine cancer. However, it is important to verify the clinical significance of FOXA1 expression in patients with apocrine breast cancer.

PSA is a 33 kD glycoprotein which was originally believed to be a tissue-specific protein produced by epithelial cells of the prostate gland. PSA is currently used as a tumor marker for prostatic adenocarcinoma. Since the transcription of PSA protein is controlled by AR, PSA expression was expected to be related to AR and FOXA1 expression. We observed a weak correlation between PSA and FOXA1 expression in this study, consistent with the role of AR and FOXA1 in PSA transcription.

Yu *et al*. reported that PSA positivity is more frequent in stage I breast tumors and declines with advancing disease stage (22). Another study showed that the expression of PSA in female breast cancer was an independent marker of good prognosis (23). In this study, we found that PSA-positive apocrine breast cancer was mostly associated with early stage and negative nodal involvement. Taken together, these results indicate that the process of carcinogenesis through the AR system takes place during an early phase in apocrine cancer.

The average age of diagnosis in patients with apocrine cancer in this study was 68.4 years, significantly higher than the average age of unselected patients with breast cancer, similar to the findings of other studies. After menopause, circulating estradiol levels decrease 10-fold due to cessation of ovulation, while testosterone levels decrease only 1.5-fold (24). In breast tissue, testosterone can potentially be metabolized by aromatase to 17-estradiol, the most potent natural ER ligand, or by 5-alpha reductase to dihydrotestosterone, the most potent natural AR ligand. After menopause, the influence of androgens may increase relative to that of estrogens. Therefore, the incidence of apocrine breast cancer affected by androgens may increase in older female patients.

From a therapeutic point of view, the finding that AR plays an oncogenic role in apocrine breast cancer provides a strong basis for therapeutically targeting AR, especially because many anti-androgenic therapies are available and approved for clinical use in the treatment of prostate cancer. Indeed, an on-going phase II clinical trial in the United States is investigating the ability of bicalutamide to stop disease progression in women with advanced $ER^-/PgR^-/AR^+$ breast cancer (www.clinicaltrials.gov, identifier NCT00468715).

In conclusion, knowledge of the immunohistochemical expression of AR, FOXA1, and PSA may play a key role in the development of targeted anticancer treatment for apocrine breast carcinoma.

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