The association between Notch4 expression, and clinicopathological characteristics and clinical outcomes in patients with breast cancer

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Received December 11, 2015; Accepted July 14, 2017

DOI: 10.3892/ol.2018.8442

Abstract. Notch4, a family member of the Notch signaling pathway, has important roles in cellular developmental pathways, including proliferation, differentiation and apoptosis. The present study aimed to investigate the association between Notch4 expression and clinical outcomes with immunohistochemistry. Notch4 was expressed in 55.6% of triple-negative breast cancer (TNBC), 45.8% of Her-2-overexpressing and 25.5% of luminal breast cancer cases, with significantly higher expression occurring in TNBC (P<0.05). Furthermore, Notch4 expression was inversely associated with estrogen receptor (ER) and/or progesterone receptor positivity, and positively associated with larger tumor size, more lymph node involvement, and more advanced tumor node metastasis stage (P<0.05). No significant association was identified regarding age, menopausal status, Her-2 status or distant metastasis. Univariate survival analysis revealed that patients with low Notch4-expressing tumors exhibited a lower relative risk of cancer recurrence compared with patients with high Notch4-expressing tumors. However, in the luminal cohort, high Notch4 expression conferred significantly lower 5-year overall survival (OS) rates compared with Notch4 low-expression groups (P=0.003) but not in TNBC and Her-2-overexpressing patients. In conclusion, Notch4 expression was significantly higher in patients with TNBC and Her-2-overexpressing breast cancer compared with luminal breast cancer patients. Notch4 expression is associated with aggressive clinicopathological and biological phenotypes, and may predict poor prognosis in luminal breast cancer patients.

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Key words: notch4 signaling, breast cancer, immunohistochemistry, prognosis

Introduction

Breast cancer is highly heterogeneous, and its biological behavior and response to therapy differ according to the subtype of breast cancer (1,2). In 2000, according to the cancer gene expression profiles, Sørlie *et al* (3) and Perou *et al* (4) divided breast cancer into luminal A, luminal B+C, human epidermal growth factor receptor 2 (Her-2)-overexpressing, basal-like and normal-like subtypes. In current clinical practice, immunohistochemical methods are used to test for estrogen receptor (ER), progesterone receptor (PR) and Her-2 expression due to the complexity, and high cost of performing molecular profiling (5-8). Patients with TNBC or Her-2-overexpressing subtypes exhibit the worst prognosis. In addition to classic prognostic factors, including tumor size, lymph nodes involved, and histological grade, some genetic and biological factors have been investigated to determine their effects on survival (9-11).

The Notch receptor family comprises four type I membrane proteins. Of them, the role of Notch4 in epithelial tumors was identified by insertional mutagenesis in mice infected with mouse mammary tumor virus (12,13). Notch4 has been identified to be expressed in stem cells of the mammary gland terminal duct, and has been implicated in the formation of branching structures that precede poorly differentiated adenocarcinoma, the restraining of TAC-2 cells to form duct branches, as well as growth factor β function, aggressive tumor phenotype, and the enabling of the transition from normal mouse mammary epithelial cells to heterotypic cells (12-15). These results demonstrated that the Notch4 signaling pathway serves an important role in the regulation of mammary gland growth and development. Abnormal expression of Notch4 may inhibit the differentiation of mammary stem cells, and mutations of the Notch4 gene may enhance mammary epithelial cell proliferation, thus leading to the occurrence of breast cancer.

In the present study, different expression levels of Notch4 were investigated in different subtypes of breast cancer. In addition, the associations between Notch4 expression, and breast cancer clinicopathological characteristics and the prognosis of patients were analyzed. Furthermore, the present study aimed to evaluate the potential of Notch4 as a prognostic marker for patients with breast cancer.

Materials and methods

A total of 98 patients who were admitted to the Cancer Hospital of Shantou University Medical College (Shantou, China) between January 1996 and December 2008 were enrolled in the current study. The study was approved by the Ethics Committee of Shantou University Medical College and written informed consent was obtained from all patients. All patients were female with a mean age of 50.5 years old (range, 36-81 years old). All patients received surgical treatment. Patients <50 years old accounted for 41.8%; 64.3% of the patients were premenopausal; 38.8% of patients had a tumor diameter reaching T1/T2, whereas 61.2% had tumor diameters reaching T3/T4; 63.3% had an N0/N1 lymph node grade, whereas 36.7% had N2/N3; 30.6% were at stage I/II, whereas 69.4% were at stage III/IV. Tumor stage was judged according to the sixth edition of the breast cancer tumor node metastasis (TNM) staging system of the American Cancer Federation (16), histological grade was judged according to the Nottingham breast cancer grading system (17). Neoadjuvant chemotherapy and adjuvant chemotherapy were all in accordance with guidelines.

ER, PR and Her-2 immunohistochemical detection was performed on all specimens as previously reported (18). The criteria for ER- and PR-positive staining was >10% of the cancer cell nuclei stained brown (19), and Her-2 was considered positive if >30% of the cancer cells presented with strong or complete cell membrane brown coloring (20). Cases were then divided into three groups according to ER, PR and Her-2 expression: i) Triple-negative breast cancer: ER, PR and Her-2 were all negative (n=27); ii) Her-2-overexpressing breast cancer: ER- and PR-negative, Her-2-positive (n=24); iii) luminal breast cancer: ER- and/or PR-positive, Her-2-negative or -positive (n=47).

Immunohistochemical staining. Formalin-fixed and paraffin-embedded breast cancer tissues were cut into 4-μm thick sections. Immunohistochemical staining was performed using Envision's two-step method to assess Notch4 expression, as previously reported (21). Briefly, tissue sections were deparaffinized with xylene and rehydrated via incubation with gradient dilutions of ethanol. Antigen retrieval was achieved through microwaving in 0.01 mol/l citrate buffer (pH=6.0) for 15 min and then allowing cooling to room temperature (RT). Endogenous peroxidase activity was subsequently blocked by incubation with 3% H_2O_2 for 10 min at RT, and then blocked with 10% normal goat serum (OriGene Technologies, Inc., Rockville, MD, USA) in PBS (pH=7.4) for 30 min at RT. Following blocking, sections were incubated with anti-Notch4 polyclonal antibody (catalog no. SC-5594; H-225; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C. Then, sections were incubated with Supervision™ Universal goat anti-rabbit horseradish peroxidase-conjugated Detection reagent (catalog no. SC-2004; Santa Cruz Biotechnology, Inc.) for 30 min at room temperature. Three washes with PBS were performed between each step of the procedure. Staining was developed with 3,3'-diaminobenzidine at room temperature and counterstained with hematoxylin at room temperature. Negative controls were evaluated by replacing the primary antibody with PBS.

Evaluation of immunohistochemistry and statistical analysis. Expression of Notch4 was primarily detected in the cytoplasm and nucleus of tumor cells, and was evaluated using a semi-quantitative scoring system as previously reported (22). Firstly, the extent of positively-labeled cells was ranked into four semi-quantitative grades: <5%, 0; 5-35%, 1; 36-70%, 2; 71-100%, 4. Secondly, the intensity of staining was categorized into four classes as follows: No staining, 0; weak staining, 1; intermediate staining, 2; and strong staining, 3. The four groups were categorized according to the multiplied score of the two classifications: Negative (-), ≤1; +, 2-3; ++, 4-5; and +++, ≥6. Based on the final score, tumor tissues that were negative (-) and weakly-positive (+) were defined as low Notch4-expressing, while tissues with moderately-(++) and strongly-positive (+++) Notch4 expression were defined as high Notch4-expressing.

All data were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The Chi-squared and two-sided Fisher's exact tests were used to assess the clinicopathological characteristics categorized by breast cancer subgroups and levels of Notch4 expression. Disease-free survival (DFS) duration was defined as the time between the date of first diagnosis and the date of the last follow-up or the date of cancer relapse. Overall survival (OS) duration was defined as the time between the date of first diagnosis and the date of the last follow-up or the date of cancer-relative death. Survival analysis was performed in sub-groups using the Kaplan-Meier survival analysis and log-rank test. Univariate and multivariate analyses were applied to quantify the effect of variables on patient survival. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Notch4 protein is expressed in the cytoplasm and nucleus of tumor cells. According to the evaluation system of Notch4-staining, 60/98 patients (61%) exhibited low Notch4 expression and 38/98 patients (39%) had high Notch4 expression (Fig. 1). Notably, high Notch4 high-expression was detected in 55.6% (15/27) of TNBC cases, in 45.8% (11/24) of Her-2-overexpression cases and in 25.2% (12/47) of luminal breast cancer cases. Patients with triple-negative or Her-2-overexpressing breast cancer exhibited significantly higher Notch4 high-expression compared with patients with the luminal type (P=0.028), but there was no significant difference between triple-negative breast cancer and Her-2-overexpressing groups (P=0.808; data not shown) (Table I).

Association between Notch4 expression and clinicopathological characteristics of breast cancer. High Notch4 expression was

Table I. Expression of Notch4 in different subtypes of breast cancer.

Breast cancer subtype	Notch4 (%)		χ^2	P-value
	Low expression	High expression		
Triple-negative	12 (44.4)	15 (55.6)		
Her-2 overexpression	13 (54.2)	11 (45.8)	7.178	0.028
Luminal	35 (74.5)	12 (25.5)		

Her-2, human epidermal growth factor receptor 2.

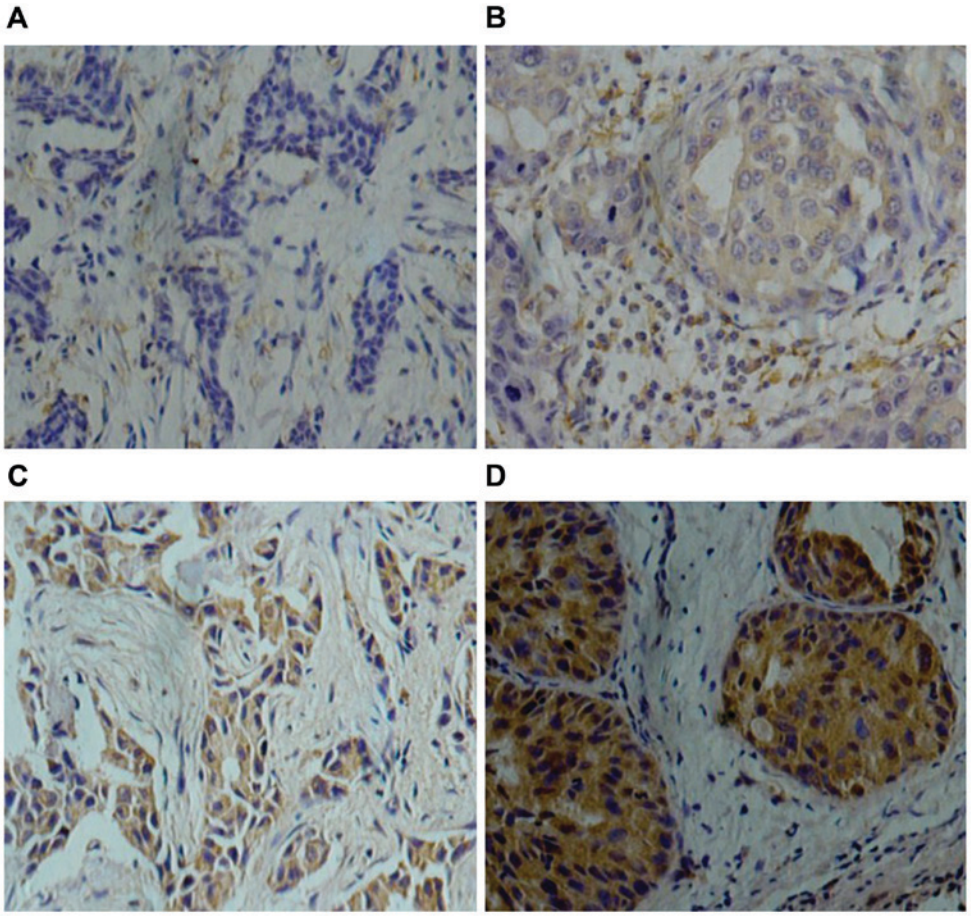

Figure 1. Immunohistochemical staining of Notch4 in breast cancer tissues (magnification, x400). (A) Negative Notch4 expression (-). (B) Low Notch4 expression (+). (C) Moderate Notch4 expression (++). (D) Strong Notch4 expression (+++).

associated with lower ER (52.8 vs. 22.2%; P=0.002) and PR (47.6 vs. 22.9%; P=0.016), larger tumor size (46.7 vs. 26.3%; P=0.044), greater lymph node metastasis (51.9 vs. 22.7%; P=0.003) and advanced TNM stage (III/IV) (47.1 vs. 20.0%; P=0.011), compared with low Notch4 expression. However, no significant difference was identified between the <50 and ≥50 years old age groups, pre- and post-menopausal groups, low and high Her-2 groups, and with or without distant metastasis (Table II).

Prognostic significance of clinicopathological factors for breast cancer. Factors, including patient age, tumor size, axillary lymph node metastasis, distant metastasis, clinical stage, ER, PR, Her-2 and Notch4 expression, were used for univariate, and multivariate analysis of OS. Univariate analysis demonstrated that patients with high Notch4 expression possessed a 3.8-fold increase in relative risk of cancer-associated mortality (95% confidence interval, 0.892-16.204; P=0.071, data not shown) compared with patients with low Notch4 expression. These results demonstrated that four variable groups: Large tumor sizes, axillary lymph node metastasis, distant metastasis present, and advanced clinical TNM stage were associated with worse prognosis (Table III).

Association between Notch4 expression and survival. The OS rates at 2, 3, and 5 years in the high Notch4-expressing group were 63.2, 36.8, and 31.6%, respectively. In addition, the 2-,

Table II. Association between Notch4 expression and clinicopathological characteristics in 98 cases of breast cancer.

Clinicopathological characteristics	Notch4 (%)		χ^2	P-value
	Low expression	High expression		
Age, years				
≤50	22 (53.7)	19 (46.3)	1.7	0.192
≥50	38 (66.7)	19 (33.3)		
Menstrual status				
Premenopausal	42 (66.7)	21 (33.3)	2.201	0.138
Postmenopausal	18 (51.4)	17 (48.6)		
ER status				
Negative	25 (47.2)	28 (52.8)	9.604	0.002
Positive	35 (77.8)	10 (22.2)		
PR status				
Negative	33 (52.4)	30 (47.6)	5.811	0.016
Positive	27 (77.1)	8 (22.9)		
Her-2 status				
Negative	37 (67.3)	18 (32.7)	1.932	0.165
Positive	23 (53.5)	20 (46.5)		
Tumor size				
T1/T2	28 (73.7)	10 (26.3)	4.059	0.044
T3/T4	32 (53.3)	28 (46.7)		
Lymph node involvement				
N0/N1	34 (77.3)	10 (22.7)	8.663	0.003
N2/N3	26 (48.1	28 (51.9)		
Distant metastasis				
No	49 (61.3)	31 (38.7)	0.009	0.925
Yes	10 (62.5)	6 (37.5)		
Tumor stage				
I/II	24 (80.0)	6 (20.0)	6.42	0.011
III/IV	36 (52.9)	32 (47.1)		

Her-2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor.

3-, and 5-year survival rates for the low Notch4 expression group were 81.8, 57.6, and 48.5%, respectively. No significant difference was identified in the OS rates between the high- and low- Notch4 expressing groups (P=0.742; Fig. 2A).

Categorization by tumor type also did not reveal a significant effect of Notch4 on OS in the TNBC or Her-2-overexpressing groups. In patients with TNBC, the 2-, 3-, and 5-year OS rates for the high Notch4 expression group were 70.0, 50.0, and 40.0%, compared with 85.7, 85.7, and 71.4% in the low Notch4 expression group (P=0.240; Fig. 2B). In patients with Her-2-overexpressing breast cancer, the 2-, 3-, and 5-year OS rates were 45.5, 27.3 and 18.2% in the high Notch4 expression group vs. 66.7, 25.0 and 16.7% in the low Notch4 expression group (χ^2, 2.408; P=0.300; Fig. 2C).

In contrast, Notch4 expression was significantly associated with a reduced survival rate in patients with luminal breast cancer. The 2-, 3-, and 5-year survival rates for the high Notch expression group were 69.7, 33.3 and 30.3%, compared with 100, 100 and 85.7% in the low Notch4 expression group. Survival was significantly lower in the high Notch4 expression group compared with in the low Notch4 expression group (P=0.003; Fig. 2D).

Discussion

In the present study, the expression level of Notch4 among different subtypes of breast cancer was explored, and the association between Notch4 expression and clinicopathological characteristics in patients with breast cancer was analyzed. The Notch4 immunohistochemical staining results reveals that Notch4 was located in the cytoplasm. Notably, cytoplasmic (perinuclear) Notch staining primarily represents newly synthesized receptors; whereas nuclear Notch staining may represent an activated receptor. Following release into nuclei, the Notch intracellular domain is rapidly phosphorylated, ubiquitinated and degraded, and seldom accumulates in the nucleus (23). Thus, the cytoplasmic expression of Notch4 detected in the present study may represent the functional protein newly synthesized.

Table III. Univariate and multivariate overall survival analyses in 98 patients with breast cancer.

	Univariate			Multivariate		
Prognostic factor	HR	95% CI	P-value	HR	95% CI	P-value
Age[a]	1.039	0.371-2.911	0.942			
Tumor size[b]	3.194	1.103-9.253	0.032			
Lymph node stage[c]	3.944	1.429-10.884	0.008			
Distant metastasis[d]	6.82	2.369-19.632	<0.001	4.421	1.502-13.012	0.007
Clinical stage[e]	6.305	1.784-22.283	0.004	3.92	1.027-14.963	0.046
ER[f]	1.131	0.420-3.043	0.808			
PR[g]	0.891	0.323-2.454	0.823			
Her-2[h]	0.723	0.249-2.094	0.550			
Notch4[i]	0.499	0.160-1.554	0.231			

[a](<50 vs. ≥50), [b]Tumor size (T1/T2 vs. T3/T4), [c]Lymph node stage (N0/N1 vs. N2/N3), [d]Distant metastasis (No vs. Yes), [e]Clinical stage (I/II vs. III/IV), [f]ER (negative vs. positive), [g]PR (negative vs. positive), [h]Her-2 (negative vs. positive), [i]Notch4 (low expression vs. high expression). HR, hazard ratio; CI, confidence interval; Her-2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor.

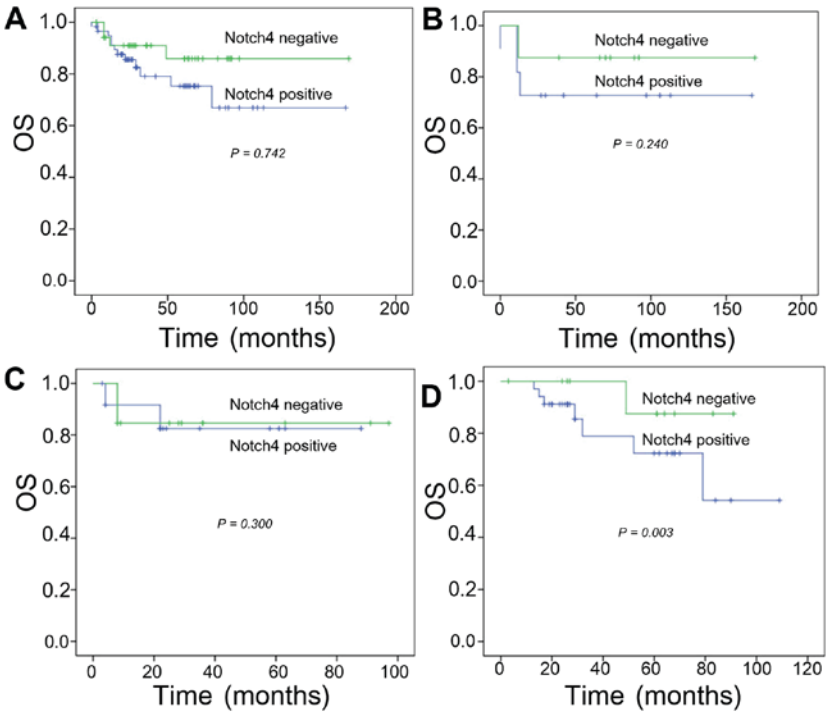

Figure 2. Comparison of OS between high and low Notch4 expression groups in the subgroups of patients with breast cancer. (A) OS analysis divided by high or low Notch4 expression groups in 98 patients with breast cancer. (B) OS analysis divided by high or low Notch4 expression in the patients with triple-negative breast cancer. (C) OS analysis divided by high or low Notch4 expression groups in patients with Her-2-overexpressed breast cancer. (D) OS analysis divided by high or low Notch4 expression groups in patients with luminal breast cancer. OS, overall survival; Her-2, human epidermal growth factor receptor 2.

Numerous studies have confirmed that the expression level of Notch receptor and its ligands in breast cancer tissue is increased compared with in normal breast tissue (24-26). For example, Rizzo *et al* (26) demonstrated that Notch1 and Notch4 expression is low in normal breast tissue, while invasive ductal carcinoma and invasive lobular carcinoma exhibited 81, and 93% Notch4-positivity, respectively. In the present study on 98 cases of breast cancer tissue, Her-2-overexpressing breast cancer and TNBC exhibited higher Notch4 high-expression compared with luminal breast cancer, which is consistent with a previous study (27). It was further demonstrated that Notch4 expression was inversely associated with ER and/or PR. Yao *et al* (27) and Rizzo *et al* (26) revealed that estrogen causes the accumulation of uncleaved Notch4 at the cell membrane while preventing Notch activation. Estrogen-treated ERα-positive breast cancer cells exhibit high levels of membrane-bound Notch, and relatively lower levels of nuclear and cytoplasmic Notch. Furthermore, Magnifico *et al* (28)

confirmed that Notch4 in Her-2-overexpressing breast cancer cells is highly active. These results supported the findings in the present study regarding the association between Notch4 expression, and ER and Her2.

In the current series of patients, Notch4 expression was identified to be inversely associated with ER and/or PR, and positively associated with tumor size, lymph node involvement and clinical TNM stage. Shawber *et al* (22) identified that Notch4 signaling is able to upregulate vascular endothelial growth factor-3 and promote cancer lymph node metastases. Yao *et al* (27) also demonstrated that cytoplasmic Notch4 expression is associated with Ki67 expression, suggesting that tumor tissues with high Notch4 expression have higher proliferation rates.

In the survival analysis, Notch4 expression does not exhibit prognostic significance in the Her-2 overexpression group. In luminal breast cancers, patients with high Notch4 expression demonstrated significantly lower OS rates compared with the low Notch4 expression group. Rizzo *et al* (26) revealed that the simultaneous use of tamoxifen and a Notch inhibitor to treat ERα-positive breast cancer cells, inhibited cell proliferation and triggered apoptosis more effectively in Notch4-expressing and ER-positive breast cancer cells. They further indicated that combinations of antiestrogens and Notch inhibitors may be effective in ERα (+) breast cancers and that Notch signaling is also a potential therapeutic target in ERα (-) breast cancers. The finding that Notch4 is able to predict the prognosis of luminal type of breast cancer suggests Notch4 may cause hormone therapy resistance, and may serve as a therapeutic target. The limitation of the present study was the relatively small number of cases included, and in certain instances, a shorter follow-up period.

In conclusion, the results of the present study demonstrated that Notch4 protein was primarily expressed in the cytoplasm in triple-negative and Her-2-overexpressing breast cancer. Notch4 expression was also identified to be inversely associated with better prognostic factors, such as small tumor size, less lymph nodes involved and positive p53 expression. In patients with luminal breast cancer, high Notch4 expression may be an important indicator and predict poor prognosis, but Notch4 is not an independent prognostic factor in patients with breast cancer. With the further understanding of its functions, the Notch4 maybe a predictor for aggressive behavior in breast cancer, and inhibition of Notch4 signaling using Notch4 antagonists may be a novel strategy to develop targeted therapy.

Acknowledgments

The present study was partially supported by the Major State Basic Research Development Program (grant no. 2011CB707705); Natural Science Foundation Committee (grant nos. 31271068 and 81302331), Major International Collaborative Research Project (grant no. 81320108015) and Guangdong Provincial Key Laboratory on Breast Cancer Diagnosis and Treatment Research.

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