Alexandria Journal of Medicine (2011) **47**, 217–224

Alexandria University Faculty of Medicine

Alexandria Journal of Medicine

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Elevated serum and tissue VEGF associated with poor outcome in breast cancer patients

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Received 7 June 2011; accepted 1 July 2011

KEYWORDS

VEGF;
HER_2 neu;
Breast cancer;
Prognosis

Abstract Vascular endothelial growth factor (VEGF) has a potent angiogenesis functions in experimental models, although their role in the progression of human breast cancer is unclear. The aim of the current study was to examine the expression pattern of VEGF in serum and tissues of breast cancer patients, examine the tumor vascular characteristic by counting the blood vessels to assess microvessles density (MVD) and conduct correlations between the expressions of growth factor in relation to patient's clinicopathological data and survival.

Methods: One hundred and twenty untreated patients with breast cancer were included in the study and followed for 4 years and 30 females with benign breast lesions matched with age and menstrual state as (control group). In this work we examine serum and tissue expression of VEGF by enzyme linked immune absorbent assay (ELISA) and immunoperoxidase technique respectively. Microvessels density were assessed and correlated with expression of growth factors.

Abbreviations: VEGF, vascular endothelial growth factor; MVD, microvessles density; ELISA, enzyme linked immune absorbent assay; HER_2/neu, human epidermal receptor.

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Peer review under responsibility of Alexandria University Faculty of Medicine.
doi:10.1016/j.ajme.2011.07.003

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Results: The mean serum level of VEGF elevated in breast cancer patients before surgery was significantly higher when compared to that in patients with benign breast lesions or in the same patient after surgery. There was positive correlation between serum and tissue VEGF. Serum and tissue vascular endothelial growth factor was strongly associated with grade III tumor, large tumor size, positive lymph node, negative hormone receptor status, +ve HER_2 neu and poor survival, the data of the present study showed significant increase in mean serum level of VEGF in patients with positive vascular invasion *P*: 0.013.

Conclusion: VEGF appear to play an important role in progression of breast carcinoma and to have significant impact on patient prognosis and can be used to identify a subset of breast cancer at higher risk for development of recurrence and distant metastasis.

1. Introduction

Breast cancer is the commonest form of cancer in women throughout the world.[1]

Angiogenesis, the formation of new blood vessels, and lymphangiogenesis, the formation of new lymphatic, are complex processes in which different signaling systems work together, one of the most potent and specific angiogenic factor is VEGF,[2] also known as vascular permeability factor and vasculotropin.[3] Evidence for the pivotal role of this cytokines in tumor angiogenesis include the observations of increased expression in tumor cells of numerous human cancers together with up regulation of the receptors on the associated endothelial cells and the inhibitory effect of anti-VEGF anti bodies on tumor growth in vivo.[4]

Higher VEGF mRNA levels have been found in invasive breast carcinoma or DCIS, compared with benign or normal breast tissue.[5,6] Assessment of VEGF expression by immunohistochemistry or immunoassay of tissue extracts has shown a significant correlation with micro vessels counts or density.[7]

Since the pivotal findings in breast cancer of a correlation between tumor angiogenesis, and metastasis,[8] many studies have confirmed the clinical value of this parameters. High mean vascular density (MVD) in breast cancer has been reported to be associated with more aggressive tumour behaviour and poor survival, intratumoral microvessels density is now considered as one of the important factors affecting survival.[9,10]

Clearly, measurement of circulating soluble marker of angiogenesis would be considerable benefit over more subjective approaches such as immunohistochemical assessments, or immunoassays which involve laborious findings of elevated VEGF concentrations in patients with cancer, many studies have reported similar findings in patients with breast cancer and many other types of cancer with a higher levels often found in metastatic disease than in localized disease or in progressive disease during treatment. Correlations with prognosis have also been reported for several cancers e.g., ovarian cancer lung cancer and colon cancer.[11–14]

The role of VEGF as a potent angiogenic factor in malignant tumors is well established, but it has long been thought that it had no influence upon lymphangiogenesis. It has recently been reported, however, that VEGF can induce lymphangiogenesis as well as angiogenesis.[15]

Most of published studies correlate angiogenesis to determine intra tumoral vascularization (or microvessel density MVD) by counting microvessels identified using immunohistochemical assays and panendothelial markers such as factor VIII, CD31, and CD34, with a recent review recommending procedures that should be followed for the assessment of MVD in breast cancer.[16]

The assessment of lymphatic characteristics in malignant tumors has historically been difficult owing to the lack of availability of lymphatic-specific markers. Such markers have recently been characterized and become commercially available. The count of positively stained vessels per tumor area, lymph vessels density (LVD), has been used to assess lymph angiogenic characteristics in tumor specimens.[16] High MVD and LVD in breast cancer have been reported to be associated with more aggressive tumor behavior and poor outcome. The aim of the current study was to investigate the expression pattern of VEGF in serum and tissues, examine the tumor vascular characteristics by counting the blood vessels to assess MVD, and conduct correlations between expression of growth factor relation to patient clinicopathological data and survival.

2. Materials and methods

Between January 2004 and June 2008, 120 non-metastatic patients with breast cancer presented and treated at the cancer management and research Department Medical Research Institute, were included in the study and followed for 4 years. Eligibility criteria were, histologically proven beast cancer, adequate haematologic parameters and normal electrocardiogram with no history of cardiac problem. All patients under went therapeutic work up including clinical history, physical examination, complete haematologic and biochemical studies, radiological studies including plain X-ray chest, abdominal ultrasound and CT scan when needed. Another 30 females with benign breast lesions matched in age and menstrual state with previous group were included as controls.

2.1. Histopathologic technique

Representative sections of 10% neutral buffered formalin fixed paraffin embedded tissue were stained with H&E stain to verify, and graded according to bloom and Richardson method.[17–19]

2.2. Assessment of MVD

Calculation of the MVD value was calculated without the aid of any immunomarker, special stain, in order to evaluate its

feasibility as a routine method in the diagnosis with the least expenses. Each section was first scanned at low-power magnification (×40) to select the most vascularized areas, three hot spots were selected. A25-point chalkley eyepieces graticule was applied to each hot spot and oriented to permit the maximum number of points to hit on or within the areas of high lighted microvessel using ×200 magnification.

Achallay count for an individual tumor was taken as the mean value of the three graticule counts.[20]

2.3. Determination of serum level of vascular endothelial growth factor (VEGF)

Human vascular endothelial growth factor (Hub VEGF) ELISA kit was used for *in vitro* quantitative analysis of human serum and it was purchased from Biosource. Camarillo, California USA.[21]

2.4. Immunohistochemistry

Four micrometer tissue sections were cut and placed on poly-lysin-coated slides and immunohistochemically stained using avindin–biotin complex immunoperoxidase technique.[22] and commercially available VEGF monoclonal anti-body. The degree of reactivity with antibody was graded semi quantitative analysis, positive tumor cells was expressed as the percentage of total number of cells, and assigned to one of four categories: Negative (0) (less than 10%), focal (+) (10–40%), variable ++ (40–70%), and uniform +++ more than (70%).

The specificity of immunohistochemical stains, in each case was confirmed by concomitant run with negative control.

2.5. Statistical analysis

Continuous parametric variable were reported as median and range. The cut-off points used for categorization were based on previously described cut-off points in the literature. Categorical variables were presented as frequency of observation and/or percentage. Correlations between categorical variables were done by Chi-square coefficient.

The duration of follow-up was calculated from the date of registration to the date of death or last follow-up. The relapse free survival period measured as the interval between the end of treatment and relapse or death or date of the last follow-up evaluation in patients who had no relapse and was estimated by Kaplan–Meier method. Overall survival period was measured as the interval between the beginning of treatment and death or date of the last follow up evaluation and was estimated by Kaplan–Meier methods.

For identification of factors that independently affecting survival we used Cox proportional-hazard model. A minimum significance level of 0.05 on univariate analysis was used as criterion for determining multivariate testing.

Statistical analysis was performed using SPSS 17, statistical package (SPSS, Inc., Chicago, Illinios).

3. Results

Table 1 showed the clinicopathological characteristics of 120 patients of non metastatic breast cancer.

3.1. Correlation of serum VEGF with clinical factors

The mean serum level of VEGF in breast cancer patients before surgery was significantly higher when either compared to that in control group $P = 0.001$ or that in the same patient after surgery. $P = 0.001$ (Table 2).

The data of the present study showed a significant increase in mean serum level of VEGF in patients with positive vascular invasion and presence of distant metastasis $P = 0.013$ (Table 3, Fig. 1). Correlation of serum VEGF and clinicopathological parameters showed, serum VEGF was strongly associated with grade III tumor, large tumor size more than 2 cm, positive lymph node, negative hormone receptor status and +ve HER2-neu (Table 4).

Table 1 Illustrate the clinicopathological characteristics of 120 patients of non metastatic breast cancer.

	Number	Percent
Age		
< 50	78	65.0
> 50	42	35.0
Range	24–72	
Mean ± SD	46.5 ±	
Menstrual status		
Pre menopause	72	60.0
Post menopause	48	40.0
Tumor size		
T1	8	6.7
T2	96	80.0
T3	16	13.3
Tumor type		
Infiltrating duct carcinoma	108	90.0
Infiltrating lobular carcinoma	12	10.0
Grade		
I	12	10.0
II	68	56.7
III	40	33.3
Vascular invasion		
Positive	24	20.0
Negative	96	80.0
Number of L.N.		
Negative	40	33.3
Positive	80	66.6
1–3	28	23.3
> 3	52	43.3
I	4	3.3
II	84	70.0
III	32	26.7
ER		
−ve	16	13.3
+ve	104	86.7
PR		
−ve	24	20.0
+ve	96	80.0
HER_2		
+ve	35	29.2
−ve	85	70.8

Table 2 VEGF (pg/ml) in control group and breast cancer patients.

		Control group no. (30)	Patient before surgery no. (120)	Patients after treatment
Range	45–280	33.0–2710.0	55–750	
Mean	125.6	450.0	172.0	
SD	52.9	108.6	103.0	
Median	130.0	460.0	175.0	
P1		0.001*	0.012*	
P2			0.001*	

Table 3 Relation between vascular invasion results, VEGF and MVD.

	Patients with negative vascular invasion	Patients with positive vascular invasion	*P*
VEGF			
Range	33–1050.0	650–2710	0.013*
Mean	330.6	960.0	
SD	205.2	805.6	
MVD			
Range	2.07–3.65	2.01–3.52	0.32
Mean	2.88	2.71	
SD	0.65	0.55	
Total number	96	24	

Table 4 Illustrate clinicopathological characteristics of patients with positive VEGF (40 patients).

	+ve VEGF
Age	
< 50	20/78
> 50	20/42
Menstrual status	
Pre menopause	25/72
Post menopause	15/48
Tumor grade	
II	14/80
III	26/40
Tumor size	
T1	0/8
T2	30/96
T3	10/16
Lymphnode status	
+ve	32/80
−ve	8/40
FR status	
+ve	16/16
−ve	24/104
PR status	
+ve	12/24
−ve	18/96
HER_2 neu	
+ve	19/35
−ve	21/85

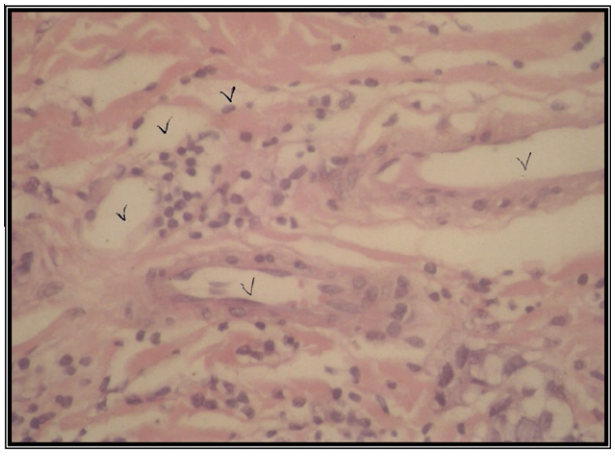

Figure 1 A case of grade III infiltrating ductal carcinoma showing a fibrovascular stroma including numerous vessels ranging from 5 to 7 vessels per high power field having MVD of 3.6 (H&E)(A-×200 and B-×400).

3.2. Survival

The minimum duration of follow up was 24 months and maximum duration 48 months calculated from the date of initiation of therapy. Survival curves were done using cox proportional hazard method. At the end of 4 years, the overall survival of patients with VEGF level above the mean was 45% versus 65% for those having VEGF concentration below the mean (Figs. 2 and 3).

In multivariate analysis, tumor size (*P* (0.001), tumor grade *P* (0.0013), number of lymph node *P* (0.0001), and VEGF *P* (0.13) were independent factors affecting overall and disease free survival (Tables 5 and 6).

3.3. Tissue expression of vascular endothelial growth factor

There was a significant association between histopathologic grade and VEGF expression *P* (0.001).

The data of ELISA (serum VEGF) correlated with the results of immunohistochemical analysis where SVEGF levels higher than median correlated strongly with uniform positive tissue expression of VEGF and correlation was significant (Figs. 4 and 5).

4. Discussion

In breast cancer, intra tumoral microvessels density IMD is now established as one of the standard prognostic factors for predicting metastasis and relapse-free or overall survival. The assessment of angiogenesis is also of potential relevance in identifying these who may benefit from anti angiogenic therapies. IMD is assessed primarily by quantification of MVD and the techniques are laborious, require experience. The measurement of circulating concentrations of specific angiogenic factors such as VEGF may provide less subjective measurement. In the present work, there was a significant association

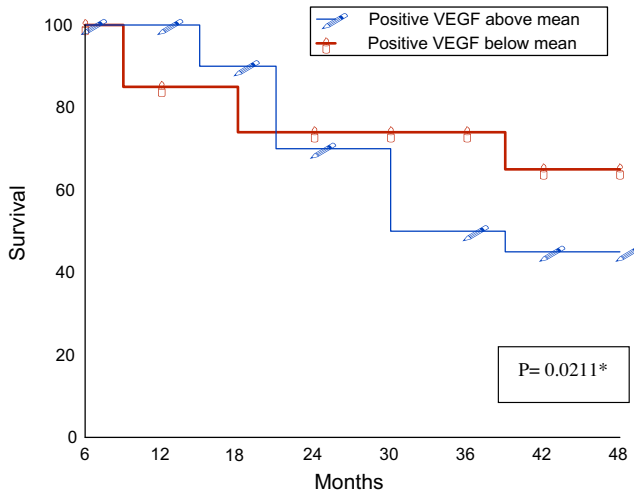

Figure 2 Overall survival of patients with non metastatic breast cancer.

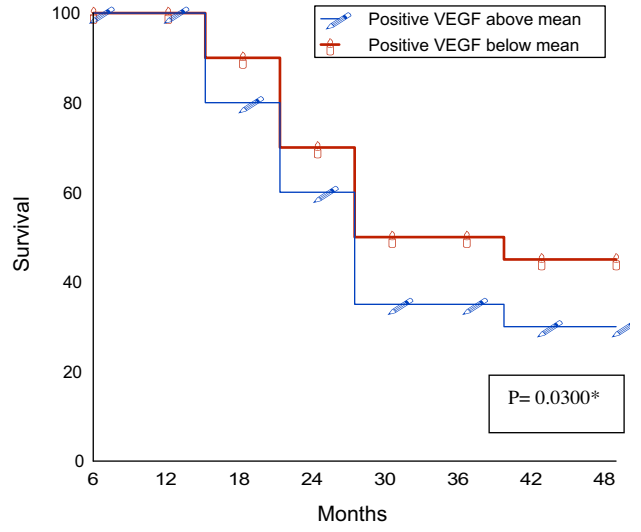

Figure 3 Disease free survival of patients with non metastatic breast cancer.

between higher serum VEGF concentration in patients with breast cancer than patients with benign breast lesions. Thus raising the possibility of using this parameter in differentiating between these two conditions.

Further more, a high serum VEGF concentration was significantly associated with high tumor grade and large tumor size more than 2 cm in size. This is agreement with previous study on breast cancer, and on lung cancer,[23] where they found that high expression of VEGF was not only associated with larger tumors but also with larger metastatic deposits, likely through the growth factor inducing a rich vascular network, and a correspondingly more nutrition environment for tumor growth. The current study also found that such tumors behaved more aggressively, as they were significantly associated with the presence of lymph nodes LN metastasis, distant metastasis and poorer survival.

Table 5 Showed different factors that affect overall survival for 48 months.

	Survive	Die	*P*
	n = 85	*n* = 35	
Age			
< 50	65	13	0.01*
> 50	20	22	
Menstrual status			
Pre menopause	52	20	> 0.05
Post menopause	33	15	
Tumor size			
T1	7	1	0.001*
T2	70	26	
T3	8	8	
Tumor type			
Infiltrating duct carcinoma	78	30	> 0.05
Infiltrating lobular carcinoma	7	5	
Grade			
I	11	1	0.0013*
II	60	8	
III	14	26	
Vascular invasion			
Positive	3	21	0.001*
Negative	82	14	
Number of L.N.			
Negative	35	5	0.001*
1–3	22	6	
> 3	28	24	
Stage			
I	4	0	0.0025*
II	70	14	
III	11	21	
ER			
−ve	10	6	0.136
+ve	75	29	
PR			
−ve	15	9	0.11
+ve	70	26	
VEGF			
Mean	420.0	920.0	0.013*
SD	102.6	465.2	
MVD			
Mean	2.64	2.45	> 0.05
SD	0.98	1.02	

These findings are similar to others, both in breast cancer and other tumor types.[24] we have also reported that low serum VEGF level was strongly associated with low stage, negative lymph-node status, and low grade tumor, findings that are compatible with those of Martin et al.[25] It has been reported that serum VEGF level changes in parallel with treatment, according to our results high serum VEGF concentrations measured before treatment were found to be correlated with high incidence of relapse, in this respect serum VEGF may be superior to the more often used breast serum markers but these findings need to be further investigated in a prospective study.

Table 6 Illustrate different factors that affect disease free survival for 48 months.

	Free Survive	Relapse or die	*P*
	n = 56	*n* = 64	
Age			
< 50	36	42	> 0.05
> 50	20	22	
Pre menopause	33	39	> 0.05
Post menopause	23	25	
Tumor size			
T1	5	3	
T2	49	47	> 0.05
T3	2	14	
Tumor type			
Infiltrating duct carcinoma	48	60	> 0.05
Infiltrating lobular carcinoma	8	4	
Grade			
I	8	4	
II	42	26	0.022*
III	6	34	
Vascular invasion			
Positive	3	21	0.013*
Negative	53	43	
Number of L.N.			
Negative	32	8	
1–3	16	12	0.001*
> 3	8	44	
Stage			
I	3	1	
II	50	34	0.013*
III	3	29	
ER			
−ve	8	8	> 0.05
+ ve	48	56	
PR			
−ve	10	14	> 0.05
+ ve	46	50	
VEGF			
Mean	325.0		
SD	109.5	465.3	0.01*
MVD			
Mean	2.65		
SD	0.88	0.66	> 0.05

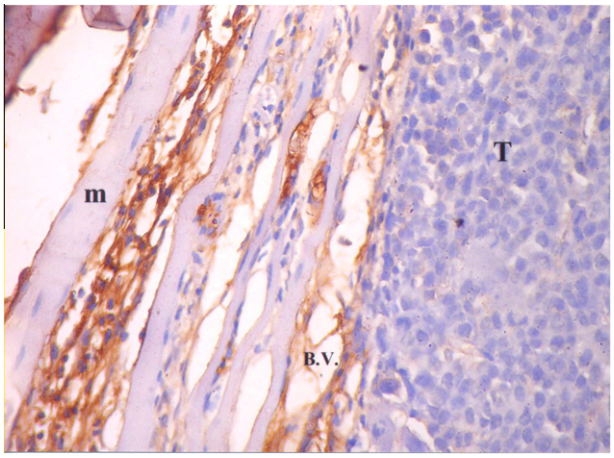

Figure 4 Breast ductal carcinoma showing malignant ductal cells (T), infiltrating part of the muscle (m), the tumor margins showing multiple, dilated proliferating blood vessels (BV) (IHC-VEGF ×100).

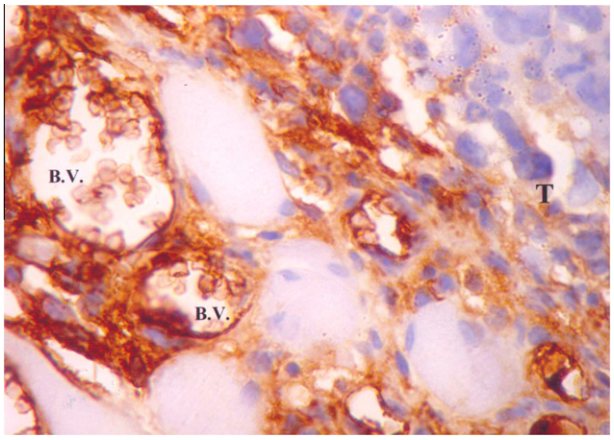

Figure 5 GIII invasive ductal carcinoma (T) showing prominent angiogenesis –multiple dilated proliferating congested blood vessels (BV) (IHC-VEGF ×400).

We have found that patients with serum VEGF level above the mean had an over all survival of 45% versus 65% for those with SVEGF level below the mean.

These data are supported by publication concerning the prognostic significance of serum VEGF[25] The 4 years over all survival rates reported in the present work were 45% for patients having their pretreatment SVEGF levels above the mean, while those with serum VEGF levels below the mean has 4 years over all survival rates 65%. In multivariate analysis SVEGF expression emerged as a significant parameter for poorer overall survival and disease free survival indicating that SVEGF is molecule particularly important for predicting worse prognosis in conjunction with other prognostic factors.

Observations that are supported by other studies[25,26] in the current study, our data identify that tissue VEGF which can be detected in archival materials, and is significantly associated with serum VEGF might be a biologically and clinically useful marker in diagnosing breast cancer and identifying high risk group.

It should be noted that breast cancer patients with positive vascular invasion have an elevated level of serum VEGF than do patients with negative vascular invasion and this supported by Altomaas et al.[27] that may reflect the importance of VEGF in vascular invasion. Since it play an important role in angiogenesis. Meanwhile, the MVD in patients with positive vascular invasion was not significantly different from patients with negative vascular invasion. Axelsson et al.[28] disagree with that and reported that, the average MVD was significantly higher in patients with vascular invasion than in patients with no vascular invasion. This can be attributed to the primitively method applied in the measurement of MVD in that study.

Overall, the clinical significance of high microvessel density in breast cancer remains uncertain, and the variability in technical approaches and difficulty in distinguishing blood and lymphatic microvessels appears to contribute to this uncertainty. Visual and image cytometric microvessel density counting methods are each associated with key advantages and limitations. For example, microscopic visual counting is less expensive and much more widely available among pathologists, but the inherent subjectivity of this method may limit inter observer reproducibility. In contrast, image cytometry is likely to be more objective and reproducible and can measure vessel luminal area, vessel luminal perimeter and the number of immunostained areas per microscopic field or scanned area. In the past few years, image cytometric microvessel area and microvessel perimeter have been demonstrated as independent prognostic factors in invasive ductal carcinoma.[29–31]

Goddard et al.[29] using anti-factor VIII to assess angiogenesis, reported no significant correlation between manual microvessel counting and computer image analysis. The Chalkley count technique seems to be preferable for estimating angiogenesis with regard to the prognostic stratification of breast cancer patients, based on its strong prognostic impact, and acceptable reproducibility.

In the present study, MVD measured by Chalkely method ranged from 1.7 to 3.9 with a mean of (2.8 ± 0.64) in breast cancer patients. Accordingly, as the tumor grade increased the MVD value increases. (MVD of G1 = 1.8, MVD of G2 = 2.5 and MVD of G3 = 3.6), these results are supported by a Chalkley count for an individual tumor when taken as the mean value of the three graticule count which resulted in that MVD ranged from 1.0 to 7.6 with a mean of (2.557 ± 0.09).

5. Conclusion

It appears from this study of human breast cancer that, as has been reported by in vitro studies, VEGF plays a role in angiogenesis. It also appear that breast cancers which express high levels of VEGF characterized by greater angiogenesis and lymphangiogenesis and are associated with the presence of both LN and distant metastasis. Such tumor behaves more aggressively as indicated by the associations with shorter disease free and overall survival. VEGF appear to play an important role in progression of breast carcinoma and to have a significant impact on patient prognosis and can be used to identify a subset of breast cancer at higher risk for development of recurrence and distant metastasis.

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