Macrophages as independent prognostic factors in small T1 breast cancers

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Abstract. Breast cancer is the second leading cause of death by cancer in women in the United States. The occurrence of high numbers of macrophages in the tumor stroma has been associated with tumor progression and poor prognosis in breast and other solid malignancies. However, macrophage numbers in tumors have not been validated as a prognostic factor in clinical practice. The present analysis was designed as a pilot study aimed at determining whether the presence of CD68+ macrophages is an independent prognostic factor in small T1 estrogen receptor (ER)⁺ breast cancers across three different ethnic groups, i.e. African-American, Latina and Caucasian women. A retrospective pilot analysis of 30 T1 breast cancer cases encompassing these three ethnic groups was carried out. African-American and Latina women present with less incidence but more aggressive breast cancer disease and, therefore, proportionally higher death rates. Using immunohistochemistry, we sought to identify whether there was any association between the presence and density of CD68+ macrophages and standard prognostic markers with overall survival in these groups. Our data revealed that overall survival did not differ significantly for the occurrence or density of CD68+ macrophages in T1 ER⁺ tumors. There were also no significant differences in overall survival for the occurrence of CD68+ macrophages across ethnicities, although macrophage numbers were significantly higher in tumors from African-American and Latina than in Caucasian patients. Importantly, but not surprisingly, the absence of the progesterone receptor was associated very strongly with decreased overall survival. This pilot project shows that CD68⁺ macrophages are not pivotal in determining tumor prognosis in early T1 breast cancers. New studies are presently being conducted to assess the value of different macrophage markers and macrophage activation profiles as prognostic factors in breast cancers of different clinical stages, using a larger number of patients among these three different ethnicities.

Introduction

Breast cancer is the most commonly diagnosed non-skin malignancy among women in the United States (US) and is the second leading cause of cancer-related death in women (1). Among prognostic factors used in clinical practice to determine the type of treatment indicated for each patient, the presence of metastatic axillary lymph nodes has been shown to be the most valuable, followed by expression of hormonal receptors [estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu)], tumor size, histological subtype, tumor grade, lymphovascular invasion and proliferative rate. Although at present, metastatic axillary node status is the single most important prognostic factor, 20% of patients with histologically negative lymph nodes suffer recurrences and die of their disease within 10 years (2). Despite the existence of several prognostic factors, it remains a clinical challenge to predict clinical outcome. For these reasons, research is ongoing to identify better or more refined tumor prognostic markers resulting in more effective treatment choices.

Additional prognostic factors have been recognized but have not yet been validated for their use in breast cancer clinical practice. Some of these include tumor DNA content, amplification of oncogenes, loss of heterozygosity (LOH) of tumor-suppressor genes, angiogenesis and expression of proteases. Moreover, new prognostic tests based on tumor gene expression profiles such as Oncotype Dx, MammaPrint, Theros, MapQuant Dx, PAM50 and Mammostrat have been recently developed and are increasingly used for the prediction of clinical outcome in breast cancer patients (3-6). An additional factor associated with poor prognosis in breast and other solid malignancies is the presence of macrophages in the

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Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2/neu, human epidermal growth factor receptor 2; IHC, immuno-histochemistry; TAMs, tumor-associated macrophages

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tumor microenvironment (7-11). There is increasing evidence that macrophages are implicated in the progression of a variety of cancers (12-14). The pro-tumor effects of tumor-associated macrophages (TAMs) include mutation induction, promotion of invasion, extracellular matrix remodeling, angiogenesis, metastasis and immune suppression (15,16).

Due to the generalized use of mammography and early detection screening programs, smaller T1 breast tumors (less than 2 centimeters in size across their widest point), are the most frequently detected in the US. When compared to Caucasian women, African-American and Latina women exhibit health disparities in breast cancer (17,18). Even though their genetic backgrounds are different, African-American and Latina women exhibit a similar pattern of breast cancer pathogenesis, with a slightly lower incidence but an earlier onset, and a more aggressive disease with less favorable prognosis (19).

Given the growing importance of macrophages in tumor progression, we examined the prognostic relevance of these cells in a small cohort of small T1 tumors across three ethnicities i.e. Caucasian, Latina and African-American women. We analyzed 30 total cases from the Sylvester Comprehensive Cancer Center Tissue Bank Core Facility's retrospective archival tumor bank. This is a tumor bank containing samples from breast cancer patients treated at Jackson Memorial Hospital and University of Miami's Sylvester Comprehensive Cancer Center in Miami, Florida between 1978 and 1996. Only T1 tumors that were ER+ by charcoal biochemical analysis (20) were selected. The charcoal method of ER determination was the one used in clinical practice to examine ER expression when these cases were diagnosed. We aimed to determine the prevalence of macrophages (CD68⁺), PR, HER2/neu and to confirm ER expression by immunohistochemistry (IHC) in these T1 breast cancers, which were all previously classified as ER+ by a charcoal biochemical method. Further, we analyzed any ethnic differences in marker staining and correlation with prognosis. We sought to evaluate whether CD68 expression in these breast cancers was associated with tumor prognosis and to assess any ethnic discrepancies between cases of Caucasian, African-American and Latina women with a pilot study of a small sample size. Our results demonstrated that macrophages are not major prognostic factors when evaluating small T1 ER⁺ breast cancers, although significant differences in TAM numbers are observed across ethnicities. Importantly, the lack of PR expression is indicative of poor tumor prognosis in this group.

Materials and methods

Case selection. Our analysis was designed as a pilot study where we randomly chose and retrospectively reviewed 30 cases of women treated for breast cancer between 1978 and 1996 at Jackson Memorial Hospital (JMH) and University of Miami's Sylvester Comprehensive Cancer Center (UM/SCCC) in Miami, Florida. Tumors were formalin-fixed paraffinembedded (FFPE) specimens from the Cancer Center's Tumor Bank Core Facility. Only the most frequently diagnosed T1 (<2 cm in size across their widest point) ER⁺ tumors were included in our analysis. The ER status at the time of the diagnosis was determined via a charcoal biochemical method (20) which was used in the clinics before the establishment of IHC methods. Patient exclusion criteria included the presence of previous cancers, exposure to chemotherapy or presenting bilateral breast cancers. Information concerning patient demographics, clinical characteristics, pathologic reports, and administered treatments was gathered from both UM/ Jackson Memorial Hospital's Tumor Registry and Medical Records and UM/SCCC. To evaluate any ethnic discrepancies in prognostic markers, we randomly selected tumor samples from 10 Caucasian, 10 African-American, and 10 Latina breast cancer patients. The present study was approved by the University of Miami's Institutional Review Board.

Immunohistochemistry. Tumors were processed by the UM FLEX System in the Department of Pathology at the University of Miami Miller School of Medicine. Tumor blocks were cut into 4- μ m sections, deparaffinized and tested by IHC for the presence of ER, PR, HER2/neu and macrophages (via CD68). Staining for ER, PR and CD68 was performed with ready-to-use IHC kits from Dako (Carpinteria, CA, USA). The HER2/ neu antibody was also from Dako, and was diluted 1:1,000. After staining, each histological sample was assessed in a blinded manner by two independent pathologists to determine ER, PR, HER2/neu and CD68 status (C.G. and M.J.).

Statistical methods. Descriptive statistics for the entire sample and for each ethnicity (Caucasian, African-American and Latina) were calculated and presented by frequencies and percentages. Chi-square or Fisher's exact tests were used for testing the equality of proportions among ethnic groups with respect to patient demographics, clinical, and pathological characteristics. Kaplan-Meier survival plots by IHC staining results were used to describe overall survival as a function of time in months. Univariate Cox regression models were fitted to identify the significant predictors of overall survival regarding PR and CD68 IHC staining results. Multivariate Cox regression models were fitted to identify the significant predictors of overall survival regarding PR and CD68 IHC staining results after adjusting for ethnicity. These regression models yielded estimated unadjusted and adjusted hazard ratios (HR) and 95% confidence intervals (CI). Wald Chi-square test derived from Cox models for testing HR were used. Statistical analyses were performed with SAS v9.2 (SAS Institute Inc., Cary, NC, USA). Type-I error rate was set to 5%, α =0.05. P<0.05 was considered to indicate a statistically significant result. Due to a small sample size, REMARK criteria for the assessment of prognostic factors were not included in this preliminary pilot study but will be used in a larger ongoing study.

Results

Tumor grade and progesterone receptor status differ between ethnicities. Of the 30 T1 cases that were gathered for our study, 29 were confirmed to be ER^+ by IHC, and only one case (a Latina woman) was determined to be ER^- by IHC, despite being previously diagnosed as ER^+ by charcoal biochemical method. This case was excluded from further analysis. Descriptive characteristics of the Caucasian, African-American and Latina breast cancer patients are provided in Table I. Mean age was 59 years (standard deviation, 12) with the youngest and oldest patient being 32 and 87 years of age, respectively. Median age was 61 with 25th and 75th percentiles of 54 and

	Total	Caucasian	Latina	African-American
No. of patients	29	10	9	10
Age (years)				
Mean (SD)	59 (12)	63 (11)	56 (7)	58 (15)
Median (Q1/Q3)	61 (54/64)	65 (62/68)	57 (52/61)	59 (54/62)
Min/max	32/87	43/77	44/63	32/87
T Stage, n (%)				
1	29 (100)	10 (100)	9 (100)	10 (100)
N Stage, n (%)				
0	16 (55)	8 (80)	3 (33)	5 (50)
1	11 (38)	2 (20)	5 (56)	4 (40)
2	2 (7)	0 (0)	1 (11)	1 (10)
M Stage, n (%)				
0	28 (97)	9 (90)	9 (100)	10 (100)
1	1 (3)	1 (10)	0 (0)	0 (0)
Grade, n (%)				
I	11 (38)	5 (50)	5 (56)	1 (10)
II	11 (38)	1 (10)	3 (33)	7 (70)
III	1 (3)	1 (10)	0 (0)	0 (0)
Not available	6 (21)	3 (30)	1 (11)	2 (20)
No. of positive nodes, n (%)				
0	16 (55)	8 (80)	3 (33)	5 (50)
1+	13 (45)	2 (20)	6 (67)	5 (50)
HER2/neu, n (%)				
Negative	29 (100)	10 (100)	9 (100)	10 (100)
ER. n (%)				
Positive	29 (100)	10 (100)	9 (100)	10 (100)
PR, n (%)				
Negative	4 (14)	1 (10)	1 (11)	2 (20)
Positive	25 (86)	9 (90)	8 (89)	8 (80)
Vital status, n (%)				
Deceased	13 (45)	4 (40)	3 (33)	6 (60)
Living	16 (55)	6 (60)	6 (67)	4 (40)

Table I. Demographics, clinical, and pathological characteristics of the women with breast cancer.

SD, standard deviation; Q1, first quartile (25th percentile); Q3, third quartile (75th percentile).

64. As delineated in our selection criteria, all patient tumors were T1 (<2 cm in size across their widest point). Only 2 (7%) cases were N2, 11 cases were N1 (38%) and the rest had no positive nodes, N0 (n=16, 55%), with 13 (45%) having one or more positive nodes. Only one case (3%) was M1. Following the criteria for tumor grading (tubular formation, nuclear pleomorphism and mitotic index) (21-23), 6 patients (21%) could not be graded, but of those who could be graded, 11 (38%) were grade I, 11 (38%) were grade II, and 1 (3%) was grade III. Statistically significant differences were noted among ethnic groups with respect to tumor grade (P=0.0236), i.e. more African-American women presented with grade II tumors than Caucasian and Latina patients. Regarding the other characteristics included in this analysis, all 29 tumors

used in the study were HER2/neu-negative by IHC and 86% were positive for PR, of which African-American cases were more likely to have had a significantly higher percentage of PR-negative cases compared with Caucasian and Latina patients. Furthermore, when the vital status was analyzed, among 29 women, 13 (45%) succumbed to the disease and 16 (55%) survived. Minimum and maximum follow-up was 24 and 292 months, respectively. Median follow-up was 138 months (~11.5 years) with 25th and 75th percentiles being 82 and 182 months (~7 and 15 years), respectively.

Macrophage numbers in the tumors are associated with ethnicity. We used two different means of assessing macro-phage staining: presence and density of CD68⁺ cells in the

	Total	Caucasian	Latina	African-American	
	n=29	n=10	n=9	n=10	
	n (%)	n (%)	n (%)	n (%)	
CD68					
Negative	8 (28)	6 (60)	1 (11)	1 (10)	
Positive	21 (72)	4 (40)	8 (89)	9 (90)	
CD68					
-	8 (28)	6 (60)	1 (11)	1 (10)	
+	4 (14)	2 (20)	2 (22)	0 (0)	
++	3 (10)	1 (10)	2 (22)	0 (0)	
+++	5 (17)	1 (10)	1 (11)	3 (30)	
++++	9 (31)	0 (0)	3 (34)	6 (60)	
CD68					
-	8 (28)	6 (60)	1 (11)	1 (10)	
+	4 (14)	2 (20)	2 (22)	0 (0)	
++/+++/++++	17 (59)	2 (20)	6 (67)	9 (90)	

Table II. CD68 staining intensity.

Table III. CD68 staining in the order of intensity by case numbers.

Caucasian		Latina		African-American	
Case no.	CD68	Case no.	CD68	Case no.	CD68
1	-	11	_	21	-
2	-	12ª	+	22	+++
3	-	13	+	23	+++
4	-	14	+	24	+++
5	-	15	++	25	++++
6	-	16	++	26	++++
7	+	17	+++	27	++++
8	+	18	++++	28	++++
9	++	19	++++	29	++++
10	+++	20	++++	30	++++
^a Excluded from the	study since FR was neg	ative			

tumors. A case was considered positive for the presence of macrophages (CD68⁺) when it exhibited any level of CD68 staining, regardless of whether it was moderate, strong or very strong. As shown in Table II, CD68 staining was available for the 29 patients. The majority of tumors (21 and 72%) were CD68-positive. Importantly, as per our analysis, of the 29 tumors, 19 (66%) were positive for both PR and CD68; in other words, out of the 25 tumors positive for PR, 19 (76%) were also positive for CD68. However, CD68 staining was significantly different among ethnicities when macrophage presence was assessed solely as positive or negative, i.e. more tumors from Caucasian women were CD68⁺ than those of African-American and Latina, and more tumors from African-American and Latina women were CD68⁺ than those of Caucasian women (Table II). These results provide evidence to conclude that there

is a relationship between the presence/absence of CD68 and the three ethnic groups (Caucasian, Latina and African-American) (Fisher's exact test, P=0.0287).

Moreover, as mentioned above, macrophage density was additionally assessed. This was arbitrarily evaluated according to the numbers of CD68⁺ cells determined in the IHC slides by two independent pathologists using the following criteria: -, no detectable expression; +, moderate expression (1-5 macrophages/slide); ++, strong expression (5-10 macrophages/slide); +++, very strong expression (10-20 macrophages/slide) and ++++, super strong expression (>20 macrophages/slide). CD68 density was also significantly different among the ethnicities (Tables II and III). When analyzing CD68 density, more tumors from African-American patients were highly populated by macrophages, followed by those of Latina women. Thus,

Table IV Overall survival



Figure 1. Photomicrograph demonstrating a representative sample of breast cancer from an African-American woman patient, using IHC staining method, and viewed at a magnification of x20. IHC showed a strong presence of macrophages.

	Overall survival (months)		
	Median	25%	75%
All patients	212	140	292
PR			
Negative	49	40	N/A
Positive	212	158	292
CD68			
Negative	140	46	203
Positive	212	182	242

All women were ER positive and HER2/neu negative.



Figure 2. Kaplan-Meier survival curves for ER, HER2/neu, PR and CD68 by IHC staining results. Women with PR⁺ tumors survival longer than those with PR⁻ tumors suggesting that the only statistically significant predictor of overall survival was PR (P=0.002); in contrast to the presence of macrophages (CD68) (P=0.210).

when instead of considering the presence or absence of macrophages, their density in the tumors was taken into account, very strong and super strong CD68 expression was noted in the vast majority of tumors from African-American and Latina patients; Caucasian women mainly showed tumors without or with few macrophages, with the exception of one patient with

Staining	HR	(95% CI)	P-value
Negative vs. positive	8.62	(1.66-44.72)	0.010
Negative vs. positive	2.06	(0.65-6.52)	0.220
- vs. ++/+++/++++	1.43	(0.41 - 4.93)	0.575
+ vs. ++/+++/++++	0.25	(0.03-2.34)	0.226
	Staining Negative vs. positive Negative vs. positive - vs. ++/+++/++++ + vs. ++/+++/++++	Staining HR Negative vs. positive 8.62 Negative vs. positive 2.06 - vs. ++/+++/++++ 1.43 + vs. ++/+++/++++ 0.25	Staining HR (95% CI) Negative vs. positive 8.62 (1.66-44.72) Negative vs. positive 2.06 (0.65-6.52) - vs. ++/+++/++++ 1.43 (0.41-4.93) + vs. ++/+++/++++ 0.25 (0.03-2.34)

Table V. Univariate Cox regression models.

Table VI. Survival rates (%).

			Years		
	0.5	1	5	10	15
All patients ^a	100	100	83	79	68
PR					
Negative	100	100	25	-	-
Positive	100	100	92	88	75
CD68					
Negative	100	100	63	63	47
Positive	100	100	91	86	76

^aAll women are ER positive and HER2/neu negative. PR, progesterone receptor. ER, estrogen receptor. HER2/neu, human epidermal growth factor receptor 2.

very strong expression and another one with strong expression. Fig. 1 shows an image of a tumor from an African-American patient with super strong presence of macrophages. This enabled us to conclude that there is an association between macrophage (CD68⁺) presence and also density within the three ethnic groups (Caucasian, Latina and African-American) (Fisher's exact test, P=0.0097), with African-American patients exhibiting the highest presence and density of TAMs.

Progesterone receptor expression is the only factor associated with clinical survival outcome. As previously shown in Table I, among the 29 women, 13 (45%) succumbed to breast cancer and 16 (55%) survived. However, a detailed description of the overall survival time for the entire sample population and by PR and CD68 is listed in Table IV. Our results revealed that women with PR+ tumors survived longer than those with PR⁻ tumors. Moreover, Kaplan-Meier survival curves for ER, HER2/neu, PR and CD68 (considering presence or absence of macrophages and not their densities in the tumors) are shown in Fig. 2. Our results suggest that the only statistically significant predictor, even in this small sample size, of overall survival was PR (P=0.002); in contrast to the presence of macrophages (CD68) (P=0.210). Furthermore, univariate Cox regression models for overall survival were fitted for dichotomous PR (positive vs. negative), dichotomous CD68 (positive vs. negative), and triple CD68 (-, +, ++/+++/+++, i.e. not detectable, moderate, strong-super strong) (Table V). HR and its 95% CI for each of the staining results were calculated. Negative PR (HR=8.62; 95% CI, 1.66-44.72; P=0.010) was significantly associated with worse overall survival. However, neither dichotomous CD68 (HR=2.06; 95% CI, 0.65-6.52; P=0.220) nor triple CD68 staining were a statistically significant predictor of overall survival (- vs. ++/+++/++++; HR=1.43; 95% CI, 0.41-4.93; P=0.575; and + vs. ++/+++/+++; HR=0.25; 95% CI, 0.03-2.34; P=0.226). Moreover, adjusting for ethnicity in the Cox regression models did not change the overall conclusion, i.e. CD68 was not a significant predictor of overall survival. In addition, Table VI shows the survival rates at 6 months and at 1, 5, 10 and 15 years. A large difference in survival at 5 years was observed for PR and not for CD68. Collectively, these data suggest that the absence of PR expression is highly associated with poor prognosis even in small T1 breast cancers.

Discussion

It has been reported that inflammatory cells in the breast tumor microenvironment, particularly macrophages, contribute to tumor progression and are associated with poor tumor prognosis (8,11). However, there is a lack of studies that focus on the prognostic relevance of macrophages in small tumors. Here we used an immune scoring detection system to identify the presence and to determine the density of macrophage infiltrates in small T1 breast cancers, in order to assess whether these inflammatory cells can be used as independent prognostic factors to predict overall survival and to aid in decisions regarding adjuvant therapy in small breast tumors. We examined the expression of the pan macrophage marker CD68 using IHC in 30 T1 early breast cancers from a retrospective tumor bank, which provided the advantage that patient outcome was known.

Significant improvements in preventive medical care in the US during the last decades, with early detection programs and mammographic screening, have resulted in the majority of breast cancers being detected as small T1 tumors. However, despite this effort, a significant percentage of these breast cancer patients with small tumors still succumb to the disease, reflecting the imperative need to develop new and more refined prognostic markers which may lead to a more precise characterization of these tumors and to more effective treatments. The current study is among one of the first designed to evaluate whether the presence and density of CD68⁺ macrophages is an independent prognostic marker in small T1 ER⁺ breast cancers across three different ethnic groups. We were particularly interested in examining whether there were any differences in macrophage distribution in breast tumors among women from different ethnicities. In particular African-American and Latina patients presented with very aggressive and poor prognosis breast cancers, as compared with Caucasian women.

Selection of breast cancer therapy is based on standard prognostic markers, such as infiltration of axillary lymph nodes, tumor size, tumor grade and expression of hormonal receptors, although more recently, gene microarray technologies have also started contributing to this decision (24-26). In the present study, we determined essential clinical and histopathological characteristics of the cases examined: primary tumor size, involvement of regional lymph nodes, expression of hormonal receptors and status of ERBB-2 protein (HER-2), and their possible inter-correlation. The initial study included tumor samples from 30 women (10 Caucasian, 10 African-American and 10 Latinas). Importantly, despite these being T1 small breast cancers, 45% of the cases had some degree of lymph node involvement (cases with N1 and N2 were included and may have confounded the result, as being the strongest clinical pathologic factor for prediction), 41% were grades II and III, and one case had a distant metastasis. However, since all these cases were still within our inclusion criteria, we did not exclude them.

Breast cancer has been historically perceived as one disease with varying histopathological features and responses to systemic treatment. In the 1970s, however, breast cancer began to be divided into two disease subsets on the basis of ER expression, in view of the distinct clinical characteristics these subgroups display (27). The most widely used technique to determine ER expression in breast tumor samples before the introduction of IHC was the Dextran charcoal assay (28). This technique was based on a multipoint saturation analysis where a fixed amount of the tumor cytosol was incubated with increasing concentrations of labeled hormone. Dextrancoated charcoal (DCC) was used to separate bound from free hormone. Yet, results obtained with this assay showed high interlaboratory variations and exhibited many inconsistencies. Although real-time PCR and cDNA microarray have been employed to determine ER status in tumors, IHC is the currently used standard method to determine ER status in clinical tumor samples. This method recognizes ER through the use of monoclonal antibodies and provides fast and highly sensitive diagnoses. Our results included tumor samples from 29 women, since out of the 30 initial ER⁺ T1 cases that were selected for our study; one case was determined to be ER- by IHC, despite being previously diagnosed as ER⁺ by charcoal biochemical method.

Although the occurrence of high numbers of macrophages in the tumor stroma has been associated with poor tumor prognosis in breast cancer in general (8), our results in small T1 breast cancers suggest no significant correlation between expression or density of the macrophage antigen CD68 and a reduced patient survival time using both univariate or multivariate survival analysis. Notably, Mahmoud *et al* (29) in a large cohort of breast cancer patients found that the presence of CD68⁺ macrophages was significantly associated with poor patient survival using univariate survival analysis, but that this association was not significant using multivariate survival analysis. Importantly, macrophage detection by IHC depends on the sensitivity of the marker used to identify this cell type. In this pilot study we used the classical pan macrophage marker CD68, which has been employed in the majority of studies. However, we are currently analyzing a larger cohort of breast cancers using the macrophage marker CD163, originally defined as an anti-inflammatory M2 macrophage marker (30) but more recently considered a pan macrophage marker, which has shown a greater sensitivity in our hands (data not shown) and in those of others (31) than CD68. Nevertheless, our present results conclude that CD68⁺ macrophage expression in small breast cancers is not a significant predictor of overall survival.

Although CD68 staining was not significantly associated with survival, we showed that the number of macrophages was significantly different among ethnicities when macrophage presence was assessed as positive or negative only, i.e. more tumors from Caucasian women were CD68-negative than those of African-American and Latina women. This result provides evidence to conclude that there is an association between the presence/absence of CD68 within the three ethnic groups. The presence of macrophages in small T1 breast tumors from African-American and Latina patients could be considered a contributive factor, among many other genetic, life style and social factors, which might in part explain the highest aggressiveness of breast cancers in African-American and Latina women. Indeed, Brown *et al* (32) demonstrated that race/ethnicity is a risk factor for survival in breast cancer.

Valuable staining information might be lost when one considers collapsing the number of macrophages to a dichotomous category as presence or absence other than considering them as macrophages i.e. ≥ 1 macrophages/slide. In our study, when instead of considering the presence or absence of macrophages, their numbers in the tumors were taken into account (as tumors with none, few, high, very high or super high numbers of macrophages), very strong and super strong CD68 expression was noted in the vast majority of tumors from African-American and Latina patients; Caucasian women mainly showed tumors without or with few macrophages, with the exception of a patient with very strong expression and another one with strong expression. Therefore, high macrophage density was also associated with ethnicity in our study, and the decision on how to properly categorize the IHC staining results should be made with great caution.

Breast cancers that are negative for ER, PR, and HER2 [called triple-negative (TN)] are associated with high-grade histology, aggressive clinical behavior and poor survival, and are highly prevalent in African-Americans and Latinas (32-34). An important finding from our study is the fact that the absence of PR expression even within small ER⁺ tumors is highly associated with poor tumor prognosis. Thus, similar to previous reports in non-T1 cases (35), the absence of PR is an independent prognostic factor for recurrence and poor clinical survival in ER⁺ breast cancer patients. ER⁺/PR⁻ tumors are a distinct subset of breast cancers characterized by aggressive behavior and tamoxifen resistance, and despite being ER⁺, they have a poor prognosis and are classified as luminal B cancers (36). Interestingly, and in contrast to our small cohort of small T1 breast cancers which were all HER2/neu-negative, the majority of these ER⁺/PR⁻ tumors were also HER2/neu⁺. It is important to point out that factors that may have limited our Cox regression analysis in the present study were the sample size and the number of events i.e. deaths. The major weakness of this pilot study was the fact that the number of the T1 tumor cases was relatively small, thus limiting subgroup analysis. Given these initial findings showing that CD68⁺ macrophages are not pivotal in determining tumor prognosis in early T1 breast cancers, new studies are presently being conducted with a larger sample size including the same three ethnicities, and comprising tumors from different stages, using various pan macrophage markers and also M1 and M2 macrophage activation markers.

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