Prognostic Value of *ERM* Gene Expression in Human Primary Breast Cancers

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ABSTRACT

We measured the expression of *ERM* gene, a nuclear transcription factor belonging to the ets family, in a series of 364 unselected primary breast cancers from patients who underwent locoregional surgery in the Centre Oscar Lambret between May 1989 and December 1991. The expression of *ERM* was quantified with a real-time one-step reverse transcription-PCR assay based on the 5′-nuclease activity of the TaqDNA polymerase and with an Abi Prism 7700 Sequence Detector System (Applied Biosystems, Courtaboeuf, France). ERM was positively correlated (Spearman test) to epidermal growth factor receptor (EGFR; $P < 0.001$, $r = 0.296$) and to histoprognostic grading ($P = 0.044$, $r = 0.112$), whereas it was negatively correlated to estradiol receptors ($P = 0.019$, $r = -0.124$), HER3 (c-erbB-3; $P = 0.01$, $r = -0.135$), and HER4 (c-erbB-4; $P = 0.003$, $r = -0.154$). Using the χ^2 test, a positive relationship was found between the expression of ERM and EGFR ($\chi^2 = 7.795$, $P = 0.007$). In overall survival studies, Cox univariate analyses demonstrated a prognostic value of ERM ($P = 0.006$; risk ratio, 2.95) besides the classical prognostic factors histoprognostic grading, node involvement, tumor size, estradiol receptors, progesterone receptors, EGFR, HER3, and HER4. In multivariate analyses, ERM preserved its prognostic value ($P = 0.004$; risk ratio, 3.779) together with histoprognostic grading, tumor size, estradiol receptors, and progesterone receptors. In relapse-free survival studies, univariate analyses demonstrated that histoprognostic grading, node involvement, tumor size, and HER4 were prognostic factors. These parameters, except histoprognostic grading, retained their prognostic value in multivariate analyses. This study demonstrates for the first time that *ERM* gene expression is an independent adverse prognostic factor for overall survival in breast cancer patients.

INTRODUCTION

Transcription factors belonging to the Ets family play an important role in a variety of physiologic and pathological processes notably in tumor development and progression (1–3). Within the Ets family, the transcription factors PEA3/E1AF/ETV4 (4, 5), ER81/ETV1 (6–8), and ERM/ETV5 (9, 10) have been assigned to the same PEA3 group according to the divergence rate analysis of the 85 residue ETS DNA-binding domain, the evolutionarily conserved region.

Several lines of evidence suggest that PEA3 group members are involved in cancer development and progression and breast cancer is the most documented (see refs. 1, 11 for review). ErbB2/HER2/neu, a member of human growth factor receptor (HER) family, is implicated in breast cancer (12, 13): relationships between PEA3 group members and HER2 have been evidenced. The overexpression of PEA3 group members was reported in primary and metastatic lesions of *neu*-induced mouse mammary adenocarcinomas (14), and the three genes are coordinately overexpressed in mammary tumors of mouse mammary tumor virus-*neu*–transgenic mice (15). Moreover, in these mice, a dominant form of the PEA3 group members delayed the onset of *neu*-induced mammary tumors and reduced their number and size (15). In the human, mRNA levels of *ERM*, *ER81*, and *PEA3* are highly elevated in estradiol receptor-negative cell lines and not in estradiol receptor-positive cells (16). Moreover, in a short series of 74 human breast carcinomas, *PEA3* mRNA is overexpressed in almost all HER2/Neu-positive tumors, whereas only half of the HER2/Neu-negative tumors overexpressed this transcription factor (17). However, in conflict with these data, a recent work reported that PEA3 overexpression can suppress HER2/Neu expression in human breast carcinoma and inhibit tumor development *in vivo*, thus prolonging animal survival and suggesting an inverse relationship between PEA3 and HER2/Neu expression (18). Moreover, a clinicopathological analysis of PEA3 protein expression in 89 breast cancer patients predicted a better overall survival in this malignancy (19).

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PEA3 overexpression is also involved in development of other tumors as lung cancer (20), oral cancer (21, 22), gastric cancer (23), colorectal cancer (24), or ovarian cancer (25). In this later study, intense expression of *PEA3* mRNA in both tumor and stromal cells is correlated with poor overall survival (25).

It has been demonstrated that proteases, as urokinase-type plasminogen activator and cathepsin D, are implicated in breast cancer progression (26–28). The different implication of PEA3 group members in physiologic and pathological processes as tumor appearance and progression is certainly because of their ability to transactivate expression of several matrix metalloproteases (21, 23, 24, 29–33), urokinase-type plasminogen activator (20, 34), as well as tissue inhibitor of metalloproteinase (32, 33), integrin subunits (25, 33), or adhesion molecules (35). These latter proteins play a critical role in the metastatic process involving angiogenesis and tumor invasion, and clearly, the pea3 group proteins play a role in their transcriptional regulation. Thus, in light of these data, it would be of great interest to evaluate the prognostic value of the expression of these transcription factors in breast carcinoma.

In the present study, we analyzed the relationships between the levels of *ERM* mRNA and the classical clinical, pathological, and biological parameters, as well as the clinical outcome in a large series of 364 primary breast cancers.

MATERIALS AND METHODS

Cell Lines. All of the cell lines were purchased from the American Type Culture Collection (Manassas, VA). MCF7, T47D, MDA-MB-231, BT20, and HBL100 cells were cultured in MEM, SK-BR-3, and MDA-MB-453 cells were cultured in RPMI 1640, and MDA-MB-468 cells were cultured in Leibovitz's L-15 medium. All media were supplemented with 10% FCS, 2 mmol/L glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and collected at subconfluence. For each cell line, two flasks of 75-cm^2 were set up.

Patients. With the agreement of the investigator's Institutional Review Board, 364 unselected breast tumor samples were obtained from patients undergoing surgery for locoregional disease in the Centre Oscar Lambret (Anticancer Center of the North of France, Lille, France) between May 1989 and December 1991 (13). The mean age of the patients was 58 years (range, 26 to 90 years). The median duration follow-up of living patients was 77.6 months. The number of deaths was 94, and the number of relapses was 126. Patients were treated by segmentectomy when the tumor was <3 cm wide and by total mastectomy if the tumor was larger or centrally located. Surgery was followed by radiation therapy. Node-positive premenopausal patients and estradiol receptor-negative and progesterone receptor (PgR)-negative postmenopausal patients received adjuvant treatment: six cycles of chemotherapy. The node-positive estradiol receptor-positive and PgR-positive postmenopausal patients received tamoxifen for 2 years. Node-negative patients received no adjuvant treatment.

Estradiol Receptor and PgR Assay. Both estradiol receptor and PgR were determined by the dextran-coated charcoal method, as described previously (36). Our laboratory is affiliated with the European Organization for Research and Treatment of Cancer Receptor Study Group, which undertook the quality control of the assays (37).

EGFR Family Member Assay. The EGFR family members (EGFR, HER2, HER3, and HER4) were quantified using a one-step real-time reverse transcription-PCR assay based on the 5′-nuclease activity of the TaqDNA polymerase and with an Abi Prism 7700 Sequence Detector System (Applied Biosystems, Courtaboeuf, France), as described previously (13).

Isolation of Total RNA. The tumor samples were frozen in liquid nitrogen and then stored at −80°C until RNA extraction. The total RNA was isolated (RNeasy Mini kit, Qiagen, Courtaboeuf, France) from 40 mg of each tumor sample and from each flask of the different cell lines. The disruption and the homogenization of the tumor samples were performed with a Rotor-Stator Homogenizer (Ribolyzer, Hybaid, Paris, France). The amount of extracted RNA was quantified by measuring the absorbance at 260 nm.

PCR Primers and TaqMan Fluorogenic Probe. The *ERM* primers and the TaqMan fluorogenic probe had the following sequences: 5′-CCAATGGGAATCAAGCAGGA-3′ (sense primer); 5′-GGATGACTGGCAGTTAGGCA-3′ (antisense primer); and 5′-CCTCGGGATTACTGCGTCGATTCAGA-3′ (probe). The size of the PCR product was 68 bp. To confirm the total gene specificity of the sequences chosen for the primers and probes, we performed nucleotide-nucleotide basic local alignment search tool searches against database of expressed sequence tags and nr (the nonredundant set of GenBank, European Molecular Biology Laboratory, and PNA data bank of Japan database sequences).

Reverse Transcription-PCR Conditions. The reverse transcription and the PCR were performed in a one-step methodology. The reaction mixture (final volume, 50 μL) contained 50 ng of total RNA, 10× (5 μL) TaqMan buffer (Eurogentec, Seraing, Belgium), 5 mmol/L $MgCl_2$, 20 units RNase inhibitor, 12.5 units of murine leukemia virus reverse transcriptase, 1.25 units of Hot Gold Star DNA polymerase (Eurogentec), 300 μmol/L deoxynucleotide triphosphate, 300 nmol/L forward and 600 nmol/L reverse primers, and 200 nmol/L probe. Reverse transcription was performed at 42°C for 30 minutes after a preliminary incubation at 65°C for 10 minutes. The activation, 10 minutes at 95°C, of the Hot Gold Star DNA polymerase was followed by PCR (15 seconds at 95°C and 1 minute at 60°C for 40 cycles). A nontemplate control was included in each experiment. The nontemplate controls and the samples were assayed in duplicate. The interassay coefficient of variation was 1.72 and 3.53% considering the Ct values for TATA box-binding protein (*TBP*) and *ERM* genes, respectively, and it was 20.78% considering the normalized expression level of *ERM* gene ($n = 9$, RNA of MDA-MB-468 cells).

Relative Quantification of ERM mRNA Expression. The quantification of PCR products is based upon the TagMan S′ nuclease assay (38, 39). To take into account the variation in RNA quantity and quality, the expression of *ERM* was normalized to a control and the endogenous *TBP* gene quantified with the primers and probe described previously (38–40). The use of *TBP* as a control RNA was relevant in these studies investigating prognosis because we observed that its expression was not associated with tumor aggressiveness (data not shown), in con-

trast with the widely used glyceraldehyde-3-phosphate dehydrogenase gene (41).

The relative quantification of *ERM* gene expression was performed with the comparative cycle threshold (C_T) method (42) where the C_T parameter is defined as the cycle number at which the fluorescent signal generated by cleavage of the dual-labeled probe is first detectable. This method is based on the use of a calibrator sample (*i.e.,* 1× sample), which permits the quantification in the unknown samples. The human breast cancer cell line MDA-MB-231, known to express *ERM*, was chosen as the calibrator sample (*i.e., ERM* expression = 1).

The relative *ERM* expression was given by the formula: $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_T$ patient sample - ΔC_T calibrator sample; with $\Delta C_T = C_{T\ ERM} - C_{T\ TBP}$.

For the $\Delta\Delta C_T$ calculation to be valid, the PCR efficiencies of *ERM* and *TBP* must be approximately equal. A method demonstrating that both have the same efficiency is to look how ΔC_T varies with template amount: the absolute value of the slope of log input amount *versus* ΔC_T should be <0.1. Before using the comparative C_T method, we performed this validation experiment to check that *TBP* and *ERM* had the same PCR efficiency. The slope passed this test (0.08).

Statistical Analyses. All of the statistical analyses were done with the SPSS software (version 11.5). Relationships between qualitative variables were determined using the χ^2 test (with Yates' correction when necessary). Correlations between parameters were assessed according to the Spearman nonparametric test. Overall survival and relapse-free survival were studied by Kaplan-Meier method analysis. Comparison between curves was carried out by the log rank test. The proportional hazard regression method of Cox (43) was used to assess the prognostic significance of parameters taken in association. No time-dependent variable was introduced.

RESULTS

Expression of *ERM* in Human Epithelial Breast Cell Lines

The expression of *ERM* was quantified in the nontumorigenic transformed human breast epithelial cell line HBL100 and in seven human breast cancer cell lines, including MCF7, T47D, MDA-MB-453, MDA-MB-468, BT20, SK-BR-3, and MDA-MB-231 used as calibrator (*ERM* expression = 1; Table 1).

ERM expression was the highest in the two cell lines BT20 and HBL100. The lowest *ERM* expression was observed in the two estradiol receptor-positive cell lines T47D and MCF7. Finally, *ERM* expression was undetectable in the two cell lines MDA-MB-453 and SK-BR-3.

Table 1 *ERM* gene expression in human epithelial breast cell lines

Cell line	*ERM* expression*
BT-20	3.63
HBL-100	3.54
MDA-MB-231	1.00
MDA-MB-468	0.35
T47D	0.05
MCF7	0.02
MDA-MB-453	ND
SK-BR-3	ND

* *ERM* expression is given relative to those of the MBA-MB-231 cells and used as calibrator (*i.e., ERM* expression = 1). Similar results were obtained from a second flask.

Abbreviation: ND, not detectable.

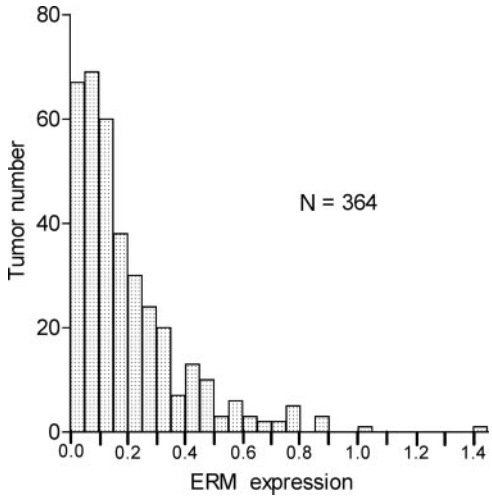

Fig. 1 Distribution of breast cancer samples as a function of their ERM expression.

Expression of *ERM* in Human Breast Cancer Biopsies

The distribution of ERM expression level in the 364 tumor samples was not Gaussian (Fig. 1). The median value was found to be 0.13 (range from 0 to 1.43). The *ERM* expression was lower than 0.05 in 67 tumor biopsies (18.4%), and it was undetectable in seven of these samples (1.9%).

Relationships with the Pathological, Clinical, and Biological Parameters

In this population of breast cancers, 73.9% of the samples were estradiol receptor positive and 72.7% were PgR positive. The classical correlations between estradiol receptor and PgR ($P < 0.001$, $r = 0.61$), estradiol receptor and age ($P < 0.001$, $r = 0.30$), and PgR and age ($P = 0.019$, $r = 0.124$) were observed. Nodal involvement strongly correlated with tumor size ($P < 0.001$, $r = 0.39$).

ERM was negatively correlated (Spearman test) to estradiol receptor ($P = 0.019$, $r = -0.124$), HER3 ($P = 0.01$, $r = -0.135$), and HER4 ($P = 0.003$, $r = -0.154$), whereas it was positively correlated to EGFR ($P < 0.001$, $r = 0.296$) and histoprognostic grading ($P = 0.044$, $r = 0.112$). Using the χ^2 test (Table 2), a strong positive relationship was found between ERM and EGFR ($\chi^2 = 7.795$, $P = 0.007$).

Prognosis Studies

Relapse-free Survival. Regardless of the threshold tested, ERM did not have prognostic value. In contrast, histoprognostic grading, node involvement, tumor size, and HER4 were prognostic factors (Table 3). As already reported, HER2 had no prognostic value in this population (13). In multivariate analyses with the parameters that have a prognostic value in univariate studies, node involvement, tumor size, and HER4 maintained their prognostic value (Table 4).

Table 2 Relation (χ^2 test) between the expression of *ERM* ($N = 364$; positivity threshold, 0.05) and clinical, histological, or biological parameters

	ERM− (<0.05)	*N** (%)	ERM+ (≥0.05)	*N* (%)	χ^2	*P*
Age (y)						
<50	15	22.4	69	23.2		
≥50	52	77.6	228	76.8	0.026	1.00
Node involvement						
Negative	28	42.4	147	49.8		
Positive	38	57.6	148	50.2	1.184	0.34
Histoprognostic grading						
I	5	7.8	30	11.6		
II	35	54.7	123	47.4		
III	24	37.5	106	41	1.366	0.505
Tumor type						
Ductular	47	70.1	204	68.7		
Lobular	9	13.4	31	10.4		
Others	11	16.5	62	20.9	1.005	0.605
Tumor diameter						
< cm	8	12.5	21	7.3		
2 to 5 cm	40	62.5	194	67.6		
>5 cm	16	25	72	25.1	1.905	0.386
Estradiol receptor						
<10 fmol/mg protein	11	16.4	83	28		
≥10 fmol/mg protein	56	83.6	214	72	2.679	0.121
PgR						
<10 fmol/mg protein	16	23.8	83	28		
≥10 fmol/mg protein	51	76.2	214	72	0.399	0.647
EGFR†						
< 0.11	44	65.6	139	46.8		
≥0.11	23	34.4	158	53.2	7.795	0.007
HER2†						
< 0.17	31	46.2	152	51.2		
≥0.17	36	53.8	145	48.8	0.492	0.502
HER3†						
< 3.45	30	44.7	152	51.2		
≥3.45	37	55.3	145	48.8	0.945	0.347
HER4†						
$< 8.5 \times 10^{-2}$	29	43.2	153	51.5		
$\geq 8.5 \times 10^{-2}$	38	56.8	144	48.5	1.544	0.226

* *N* indicates the number of patients.
† The thresholds of EGFR, HER2, HER3, and HER4 are the respective median values as reported previously (13).

Overall Survival. The best ERM threshold for prognosis was 0.05; nevertheless, a threshold of 0.04 also allowed us to distinguish two populations of different prognosis. Shorter overall survival was found in patients with elevated *ERM* expression (Fig. 2). The number of death was ~3-fold higher in ERM^+-patients than in ERM^--patients (87 of 297 *versus* 7 of 67). Histoprognostic grading, node involvement, tumor size, estradiol receptor, PgR, EGFR, HER3, and HER4 were also prognostic factors (Table 3). As already reported, HER2 had no prognostic value in this population (13). The prognostic signif-

Table 3 Prognostic factors in Cox univariate analyses

	Overall survival		Relapse-free survival	
	P	Risk ratio	*P*	Risk ratio
ERM (< 0.05; ≥0.05)	0.006	2.95	NS	NS
Histoprognostic grading (I, II, III)	0.001	1.837	0.029	1.38
Node involvement (0; >0)	0.034	1.564	0.009	1.615
Tumor diameter (≤2; 2 to 5; >5 cm)	0.004	1.749	0.002	1.666
Estradiol receptor (< 10; ≥10 fmol/mg protein)	0.044	0.642	NS	NS
PgR (<10; ≥10 fmol/mg protein)	0.004	0.543	NS	NS
EGFR (<0.11; ≥0.11)*	0.026	1.604	NS	NS
HER3 (<3.45; ≥3.45)*	0.009	0.577	NS	NS
HER4 ($<8.5 \times 10^{-2}$; $\geq 8.5 \times 10^{-2}$)*	0.002	0.524	0.015	0.646

* The thresholds of EGFR, HER3, and HER4 are the respective median values as reported previously (13).
Abbreviation: NS, not significant.

Table 4 Prognostic factors in Cox multivariate analyses

	Overall survival		Relapse-free survival	
	P	Risk ratio	*P*	Risk ratio
ERM (< 0.05; ≥0.05)	0.004	3.779	NS	NS
Histoprognostic grading (I, II, III)	0.038	1.585	NS	NS
Node involvement (0; >0)	NS	NS	0.047	1.485
Tumor diameter (≤2; 2 to 5; >5 cm)	0.012	1.77	0.013	1.571
Estradiol receptor (< 10; ≥10 fmol/mg protein)	0.012	0.674	NS	NS
PgR (< 10; ≥10 fmol/mg protein)	0.006	0.409	NS	NS
EGFR (<0.11; ≥0.11)*	NS	NS	NS	NS
HER3 (<3.45; ≥3.45)*	NS	NS	NS	NS
HER4 ($<8.5 \times 10^{-2}$; $\geq 8.5 \times 10^{-2}$)*	NS	NS	0.043	0.640

NOTE. The parameters that had a prognostic value in the univariate analyses were entered in this study.
* The thresholds of EGFR, HER3, and HER4 are the respective median values as reported previously (13).

icance of ERM was assessed separately in lymph node-positive ($N = 186$) and lymph node-negative ($N = 175$) patients, and ERM was found to be a prognostic factor on overall survival ($P = 0.0155$) in lymph node-positive patients. In multivariate analyses, when combining the parameters that have a prognostic value in univariate analyses, ERM preserved its prognostic value ($P = 0.004$; risk ratio, 3.779) together with histoprognostic grading, tumor size, estradiol receptor, and PgR (Table 4).

DISCUSSION

In this study, the expression of *ERM* gene is analyzed for the first time in a large series of primary breast cancers to establish the relationships between expression and the pathological, clinical, and biological parameters and the clinical outcome. The expression was quantified with a real-time reverse transcription-PCR assay as described previously (13).

In the eight analyzed cell lines, the expression of *ERM* was found to be in agreement with our previous observations demonstrating that BT-20, HBL-100 and MDA-MB-231 cells express the highest levels of *ERM* (16).

With respect to the clinical, histologic, and biological parameters, elevated *ERM* expression was positively correlated to histoprognostic grading and inversely correlated to the estradiol receptor. Such results suggest that *ERM*-elevated mRNA expression could be a biological marker of a less differentiated phenotype. Similar results were observed studying PEA3, another Ets-related transcription factor (44). Interestingly, using cDNA array analysis, *ERM* has been detected as one of the genes showing the most frequent differential expression between normal breast tissue and breast cancer, with its expression being enhanced (3.6-fold) in tumors (45).

In the same population of patients, we previously assayed the expression of EGFR family members (13): we confirmed that EGFR and HER2 were markers of tumor aggressiveness and demonstrated that HER3 and HER4 were markers of differentiation. We presently evidence negative correlations between ERM and HER3 and HER4. In contrast, a strong positive correlation between ERM and EGFR is pointed out. Number of studies has been published concerning the relations between HER2 and PEA3. PEA3 was found increased in HER2-positive experimental tumors (14) or breast cancers (17). It has been also suggested that *PEA3* could be a tumor suppressor gene repressing HER2 (18). With the present results, it is difficult to explain the correlation between ERM and EGFR, but we cannot exclude the hypothesis that the increase in ERM would result from the increase in EGFR, with *ERM* acting as a suppressor gene.

We found that ERM had a prognostic value in terms of overall survival in univariate analyses. Recently, PEA3 prognostic value was found to be either positive (19) or absent (44) in small series of breast cancers. We present the first report demonstrating a prognostic value of a Ets-related transcription factor in a large series of breast cancers. Logically, as ERM is related to poor prognosis parameters, it is associated with poor prognosis. The ability of this gene to transactivate matrix metalloproteases (20, 21, 29–34) is certainly an important factor of this association. Finally, the multivariate Cox analyses combining ERM, EGFR, HER3, HER4, and the other classical biological and clinical prognostic factors revealed that ERM maintained its prognostic value on OS together with the classical prognostic factors such as tumor size, histoprognostic grading, estradiol receptor, and PgR. Nodal status did not maintained its prognostic value. Such a result is certainly due to the fact that in the studied population, nodal involvement strongly correlated

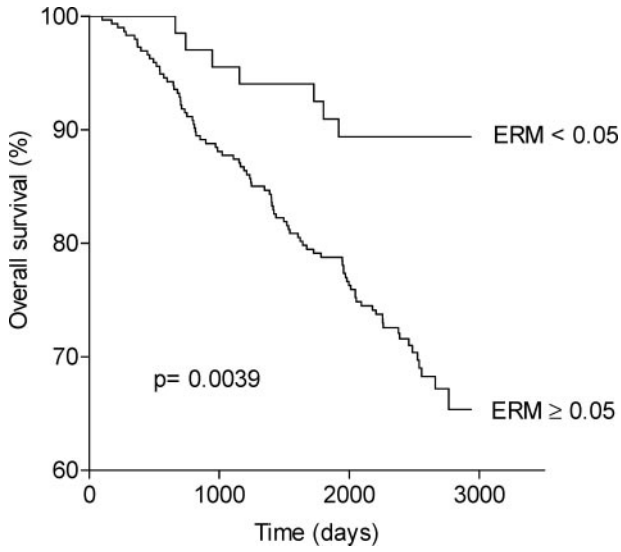

Fig. 2 Kaplan-Meier plots of overall survival according to the expression of *ERM*.

with tumor size. Therefore, tumor size functioned as a surrogate for nodal status in the multivariate model as we already observed combining other parameters (13).

In conclusion, the present study demonstrates that the expression of ERM is a marker of tumor aggressiveness in breast cancer.

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