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Circulating transforming growth factor- β -1 and breast cancer prognosis: results from the Shanghai Breast Cancer Study

Ana M. Grau,

Department of Surgery, Division of Surgical Oncology and Endocrine Surgery, Vanderbilt University, 2220 Pierce Ave, 597-PRB, Nashville, TN 37232-6860, USA

Wanqing Wen,

Department of Medicine, Vanderbilt Epidemiology Center, Vanderbilt-Ingram Cancer Center, Vanderbilt University, S-1121 MCN, 1161 21st Ave. S, Nashville, TN 37232-2587, USA

Denise S. Ramroopsingh,

Department of Surgery, Meharry Medical College, Nashville, TN, USA

Yu-Tang Gao,

Department of Epidemiology, Shanghai Cancer Institute, Shanghai, China

Jinghuan Zi,

Department of Surgery, Division of Surgical Oncology and Endocrine Surgery, Vanderbilt University, 2220 Pierce Ave, 597-PRB, Nashville, TN 37232-6860, USA

Qiuyin Cai,

Department of Medicine, Vanderbilt Epidemiology Center, Vanderbilt-Ingram Cancer Center, Vanderbilt University, S-1121 MCN, 1161 21st Ave. S, Nashville, TN 37232-2587, USA

Xiao-Ou Shu, and

Department of Medicine, Vanderbilt Epidemiology Center, Vanderbilt-Ingram Cancer Center, Vanderbilt University, S-1121 MCN, 1161 21st Ave. S, Nashville, TN 37232-2587, USA

Wei Zheng

Department of Medicine, Vanderbilt Epidemiology Center, Vanderbilt-Ingram Cancer Center, Vanderbilt University, S-1121 MCN, 1161 21st Ave. S, Nashville, TN 37232-2587, USA

Abstract

Introduction—Studies investigating the prognostic effect of circulating TGF- β -1 in breast cancer have given inconsistent findings. The purpose of this study is to evaluate whether circulating transforming growth factor beta 1 (TGF- β -1) is associated with overall and disease-free survival in a cohort of recently diagnosed breast cancer patients.

Methods—We measured TGF- β -1 levels in plasma samples of breast cancer patients in the Shanghai Breast Cancer Study, a population-based case-control study. We evaluated the relationship between TGF- β -1 levels and overall and disease-free survival. The median follow up time was 7.2 years.

[✉] ana.grau@vanderbilt.edu.

Results—We observed that, compared with the patients with the lowest quartile of plasma TGF- β -1, patients with the highest quartile of plasma TGF- β -1 had significantly worse overall survival with hazards ratio (HR) = 2.78, with 95% confidence interval (CI): 1.34–5.79 and disease-free survival with HR = 2.49, 95% CI: 1.15–5.41, while the patients with the second and third quartiles of plasma TGF- β -1 did not have significantly different overall and disease-free breast cancer survival. The shape of association between plasma TGF- β -1 levels and breast cancer survival appears to be nonlinear. Stratified analysis by stage of disease did not appreciably change the association pattern.

Conclusions—We conclude that the relationship between circulating levels of TGF- β -1 and prognosis in breast cancer is complex and nonlinear. High levels of TGF- β -1 are associated with worse survival independent of stage of disease.

Keywords

TGF- β 1; Plasma; Breast cancer; Overall survival; Disease-free survival

Introduction

Abnormalities in the transforming growth factor β (TGF- β) signal transduction pathways are involved in the promotion of cancer in humans. TGF- β is a family of hormonally active polypeptides that inhibit the proliferation of normal epithelial cells, and enhance matrix formation as well as angiogenesis [1]. It is known that most epithelial cancer cells lose responsiveness to TGF- β induced growth inhibition [2, 3]. Loss of TGF- β responsiveness may be due to alterations on the TGF- β serine/threonine kinase receptors (receptors type I and II) or due to inactivating mutations of smad4, a candidate tumor suppressor gene implicated in breast, pancreatic and colonic tumorigenesis [4]. P21waf1, a cyclin dependent kinase inhibitor, has been identified as one of the smad4 target genes [3], and overexpression of smad4 induces both p21waf1 expression and growth inhibition in human carcinoma cells [5]. TGF β is involved in the induction of epithelial-mesenchymal transition (EMT) and migration [6], angiogenesis and reduction of immunogenicity [7]. As cancer develops, both cancer cells, as well as adjacent stromal cells increase their production of TGF- β resulting in a paracrine effect [8, 9]. Recent data indicates that breast cancer cells can secrete TGF- β -1 and induce the expression of matrix-metalloproteinases in stromal cells [10], and that TGF- β -1 produced by tumors significantly reduces the efficacy of dendritic cell/tumor fusion vaccine [11].

TGF- β has three isoforms, TGF- β -1, TGF- β -2, and TGF- β -3. Each isoform is encoded by a distinct gene and has tissue-specific expression, with TGF- β -1 being the predominant isoform found in blood [9, 12]. TGF- β -1 levels have been associated with worse cancer prognosis. In a prospective study of 117 patients with colorectal carcinoma, the circulating levels of TGF- β -1 before and after curative resection were associated with progression of disease and liver metastasis [13]. Similar findings have been observed for breast, prostate, and hepatocellular cancer [14–17]. Wakefield et al. [12] suggested a potential endocrine role for TGF- β -1 in breast cancer. They observed that TGF- β -1 is the predominant TGF- β isoform in plasma, although they did not observe a difference in plasma TGF- β -1 between normal human subjects and 26 out of 28 patients with advanced metastatic breast cancer

[12]. In a study of 26 newly diagnosed breast cancers, Kong et al. observed that breast tumors result in increased plasma TGF- β -1 levels in 81% of patients compared with healthy controls. After surgical removal of the primary tumor, the plasma TGF- β -1 level receded in the majority of patients; persistently elevated levels were correlated with the presence of lymph node metastases or overt residual tumor [18]. Mixed findings were reported on the association between circulating TGF- β -1 levels and TNM stage, histological grade or estrogen receptor status [19–21].

Elevated circulating levels of TGF- β -1 was found to be related with disease progression in breast cancer [14]. One study found that plasma TGF- β -1 levels were similar in stages I/II breast cancer patients and healthy controls, however, stage IV breast cancer patients had a significant elevated plasma TGF- β -1. Moreover, patients with elevated plasma TGF- β -1 had a worse breast cancer prognosis [22]. The same authors have reported that, in patients with metastatic disease, TGF- β -1 elevation was independent of tumor mass [14].

In this study, we investigated the association of plasma TGF- β -1 with breast cancer survival using data collected from the Shanghai Breast Cancer Study in an effort to define the role of circulating TGF- β -1 as a prognostic factor in breast cancer. In particular, we studied the relationship between pretreatment plasma TGF- β -1 levels and stage of disease given the previously discussed suggested role of tumor burden as the origin of circulating TGF- β -1, and whether pretreatment plasma TGF- β -1 levels can predict survival independent of tumor stage.

Methods

Participants and study design

Participants in the study were breast cancer patients who were recruited to the Shanghai Breast Cancer Study [23], a population-based case-control study. Details of the study have been described elsewhere [23]. In brief, through a rapid case-ascertainment system, supplemented by the population-based Shanghai Cancer Registry, 1,602 newly diagnosed breast cancer cases between the ages of 25 and 64 during the period from August 1996 to March 1998 were identified. In-person interviews were completed for 1,459 (91.1%) of them. The major reasons for non-participation were refusal (109 cases, 6.8%), death prior to interview (17 cases, 1.1%), and inability to locate (17 cases, 1.1%). All diagnoses were confirmed by two senior pathologists through the review of slides. All study participants were interviewed by trained interviewers at hospitals or at home. A structured questionnaire was used to elicit detailed information on demographic factors, menstrual and reproductive history, hormone use, dietary habits, prior disease history, physical activity, tobacco and alcohol use, weight history, and family history of cancer. Information on cancer diagnosis, disease stage, cancer treatments, and estrogen and progesterone receptor status was abstracted from medical charts using a standard protocol.

Of all 1,459 breast cancer patients, 1,455 were followed until July 2005 for cancer recurrence and mortality with a combination of two active follow-up surveys and record linkage to death certificates kept by the Vital Statistics Unit of the Shanghai Center for Disease Control and Prevention. The median follow-up time for the cohort was 7.2 years.

Through interview of patients, or next of kin for deceased patients, we obtained information on disease progression, recurrence, quality of life, and cause of death (if deceased). Of the 1,455 eligible patients, 1,378 were followed-up via in-person contact or by phone (active follow-up) at least once during the two follow-ups. Survival status for the remaining 77 participants was established by linkage to the death registry in July 2005. A peripheral blood sample (10 ml from each woman) was obtained from 1,193. Since there is evidence that blood TGF- β -1 levels can be affected by surgery, chemotherapy and radiation therapy [24], we wanted to study the levels before any cancer treatment. Included in the current study were all participants ($n = 439$) for which blood samples were collected *prior to any treatment* (i.e., surgery, chemotherapy, and radiation therapy). Figure 1 describes the flow of study participants, reasons for dropout, and inclusion criteria. Venous blood was obtained using Vacutainer EDTA tube. The blood samples were processed for plasma within 6 h of collection and stored at -70°C in aliquots. All samples were handled uniformly, and they were subject to one freeze and thaw cycle before assays were performed.

Laboratory assays

An enzyme-linked immunosorbent assay (ELISA) Quantikine® TGF- β -1 kit from R&D Systems (Minneapolis, MN) was used for determination of plasma TGF- β -1 following the manufacturer's instructions. Before assay, plasma for TGF- β -1 measurement was activated (according to the manufacturer's instructions) with 1 N HCl and neutralized with 1.2 N NaOH/ 0.5 M HEPES, to release the active cytokine from the latent complex. The intraassay variation in our hands was 7.3–7.7% and the interassay reproducibility was 3.6–10.7%. The minimum detectable TGF- β -1 level was 8 pg/ml. The recovery was 83–126% and linearity 83–109%. These numbers were similar and comparable with to the manufacturer's description. Measurements were performed in triplicate, and the average of these three measures was used to calculate TGF- β -1 levels. The assays were performed blinded to the study endpoints.

Statistical analysis

The association between plasma TGF- β -1 levels and Overall and Disease-free breast cancer survival was evaluated using hazard ratios (HR) and 95% confidence intervals (CI) derived from a Cox regression model with plasma TGF- β levels being categorized into quartiles and with AJCC TNM (American Joint Committee on Cancer staging) stages and history of radiotherapy being adjusted for in the model. Missing variables were treated as separate categories in this model. Additional adjustment for age at diagnosis, menopausal status, education levels and ER/PR status produced little changes on the estimates, thus, the final models only included TNM and radiotherapy as potential confounders.

To evaluate the association shape between the continuous plasma TGF- β -1 levels and the breast cancer survival, non-linear terms were included in the model using the restricted cubic spline function with four knots [25]. REMARK criteria for tumor marker studies was used for the preparation of this manuscript [26].

Results

Table 1 shows the overall survival by demographics and known breast cancer prognostic factors for 439 breast cancer participants in the Shanghai Breast Cancer Study for which we measured pre-treatment plasma TGF- β -1 levels. As expected, advanced TNM stages were related with worse overall breast cancer survival. Age, education level, menopausal status, and estrogen and progesterone (ER/PR) status were not found to be significantly associated with overall breast cancer survival. The association of these demographic factors, TNM stage, and clinical parameters with overall survival was similar in the patients with TGF- β -1 data and patients in the whole survival study [27]. Similar results were observed for disease-free survival (data not shown).

The mean of pre-treatment plasma TGF- β -1 in the 439 breast cancer patients was 8.76 ng/ml with a range of 0.130 to 37.99 ng/ml. The plasma levels were not significantly different between 116 patients with stages 0 and I and 311 patients with stage II or above and not related with estrogen receptor status. The levels were 8.70 ng/ml with a range of 0.58 to 37.99 ng/ml for 116 patients with stages 0 and I and 8.56 ng/ml with a range of 0.13 to 30.08 ng/ml for 311 patients with stage II or above. The difference of mean plasma TGF- β -1 between those two subgroups of patients was not statistically significant ($P = 0.820$).

Presented in Table 2 were HR and 95% CI for the association between pre-treatment TGF- β -1 plasma levels by quartiles and breast cancer overall survival and disease-free survival. Compared with the patients with the first (lowest) quartile of plasma TGF- β -1 (0.130–4.764 ng/ml), the patients with the second and third quartiles did not have significantly different overall breast cancer survival, while the patients with the highest quartile of plasma TGF- β -1 (11.082–37.99 ng/ml) had significantly worse overall survival (HR = 2.78, 95% CI: 1.34–5.79), even after adjusting for TNM stage and radiotherapy (Table 2-among all patients). Stratified by TNM stage (0, I, IIa vs. IIb, III, IV), a similar association pattern held. Similarly, TGF- β -1 plasma levels above 11 ng/ml (quartile 4) were associated with worse disease-free survival (HR = 2.49, 95% CI: 1.15–5.41). Stratified analysis by TNM stages did not dramatically change the association pattern, although the overall survival and disease free survival associated with the highest quartile of plasma TGF- β -1 seemed to be worse in breast cancer patients with higher TNM stages than in those with lower TNM stages.

Figure 2a, b demonstrate the shapes of the association of plasma TGF- β -1 levels with the overall and disease-free survival of breast cancer patients, respectively, with use of the restricted cubic spline function with four knots. A non-linear relationship is apparent (P for non-linearity = 0.043 for both overall and disease-free survival).

Discussion

Our study, thus far the largest one on the topic, suggested a prognostic role of pre-treatment circulating TGF- β -1 levels. Using measurements in plasma, our results suggest a complex, non-linear relationship between pre-treatment TGF- β -1 levels and survival in breast cancer that is statistically significant. The association between pre-treatment TGF- β -1 plasma levels was seen when analyzing for both overall as well as disease-free survival, an important

observation since elevated circulating TGF- β -1 levels have been observed in relation to other chronic, mostly cardiovascular and renal, illnesses [28] that could have a more pronounced impact on overall survival than disease-free survival. Of note, the Shanghai Breast Cancer study cohort is characterized by a low rate of these cardiovascular diseases and risk factors [29].

Prior studies have investigated the prognostic effect of circulating TGF- β -1 in breast cancer with inconsistent findings. While no relation between circulating TGF- β -1 levels and tumor progression and/or stage and tumor grade [30], stage of disease [12, 21], and lymph node and hormonal receptor status [20] were found in some studies, other studies have reported a positive relation between higher circulating levels and advanced stage at diagnosis [14, 18, 19], high risk for stage IV, ER-, PR- breast cancer patients [31], and, in the most recent study, a worse 2-year overall survival for stage IV breast cancer patients [22]. Both plasma [12, 18, 21, 22, 30, 31] and serum [19, 20] have been used as collection formats in previous studies. It does not appear that the sample collection methods (plasma vs. serum) influence study results with regards to the relation of TGF- β -1 with tumor stage and progression. Shariat et al [16] studied the impact of collection formats on TGF- β -1 levels and found that TGF- β -1 levels measured in serum were higher than plasma. They hypothesized that since TGF- β -1 was present in platelets granules and was released upon platelet activation, it would make the quantification of non-platelet TGF- β -1 less accurate. This observation has been confirmed by others [32]. It is for this reason that we elected to use plasma as our collection format.

Some studies suggested a potential for tumor burden as the source of increased circulating TGF- β -1, since TGF- β -1 levels have been found to be elevated in breast cancer tissues [33]. Kong et al. [18], in their study of 26 breast cancer patients, observed that TGF- β -1 plasma levels decreased after tumor resection. Conversely, others have observed that elevation of plasma TGF- β -1 levels in advanced breast cancer patients is independent of tumor mass [14]. It is likely that the determinants of circulating TGF- β -1 are multifactorial. Our study cannot answer that question; however, the distribution of patients by TGF- β -1 quartiles between earlier and more advanced stages of disease seen in Table 2, as well as the lack of statistical difference in TGF- β -1 levels between stage 0–I and stage II–IV patients, do not support tumor burden as the *only* source of elevated circulating TGF- β -1.

The circulating TGF- β levels may be under genetic control [34]. Several polymorphisms of the TGF- β -1 gene have been observed (reviewed by Yokota et al.) [35]. The T29C polymorphism was found to be associated with circulating levels of TGF- β -1 in serum [35] and C-509T polymorphism was found to be associated with circulating levels of TGF- β -1 in plasma [34]. Our previous study showed that patients who carried the C allele of T29C polymorphism had a reduced 5-year breast cancer disease-free survival in the Shanghai Breast Cancer Study [36].

The potential effect of circulating TGF- β -1 in human breast cancer is still unclear. Results from a breast cancer animal model study indicated that post treatment elevations of circulating TGF- β -1 might have an impact in tumor progression, and that blockade of TGF- β -1 effect results in decreased breast cancer lung metastasis in this model [24], suggesting

that there could be a potential role for therapeutic interventions aimed at reducing the circulating levels of TGF- β -1 [8, 37]. The role of TGF- β -1 in cancer is complex and context-dependent, with a predominant tumor suppressor role in normal breast and in some breast cancer cells. As tumors progress, TGF- β -1 may actually promote tumorigenesis by its direct effect on cancer cells and their stromal microenvironment [9]. Whether this known contextual effect of TGF- β -1 may help explain its suggested non-linear relationship with survival in our breast cancer patients is a hypothesis that the present study is not designed to address.

The strengths of our study include a relatively large sample size, population-based patient setting, high response rate, and high follow-up rate. Information on stage of disease and treatment was obtained for a high proportion of patients, and we had a relatively large number of patients for which pre-treatment plasma was available. However, in our study blood was processed within 6 h of collection, not necessarily immediately after collection. This may have allowed for some variability in platelet degranulation between samples and may partly explain the wide range in TGF- β -1 levels.

In summary, our data shows that pre-treatment circulating plasma levels of TGF- β -1 in the highest quartile were related with significantly worse overall and diseases-free survival, even after controlling for stage of disease. With levels of TGF- β -1 in plasma not necessarily representing simple tumor TGF- β -1 spillage into the circulation, it is conceivable that levels of TGF- β -1 in blood may determine prognosis because of its effects on tumor progression locally and/or systemically, as opposed to merely representing a secondary manifestation of tumor burden. This could position circulating TGF- β -1 as a potential target for cancer therapy.

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Fig. 1.
Flow of SBCS participants including dropout reasons and inclusion criteria

Fig. 2.

(a) The log (HR) (*solid line*) and 95% confidence limits (*dash lines*) for the association between plasma TGF- β -1 levels and the overall survival of breast cancer patients (adjusted to TNM and radiotherapy). P for non-linearity = 0.043. (b) The log (HR) (*solid line*) and 95% confidence limits (*dash lines*) for the association between plasma TGF- β -1 levels and the disease-free survival of breast cancer patients (adjusted to TNM and radiotherapy). P for non-linearity = 0.043

Table 1

Overall survival by demographics and known breast cancer prognostic factors, the Shanghai Breast Cancer Study

	Total	No. death	3-year survival(%)	P
Age				
<45	170	21	88.2	0.653
45–49	126	14	88.9	
50	143	21	85.3	
Menopausal status				
Pre-	315	38	88.3	0.579
Post-	121	17	86.0	
Education levels				
<High school	252	29	88.9	0.373
High school	187	27	85.6	
ER				
Positive	231	28	88.3	0.293
Negative	101	10	90.1	
PR				
Positive	234	29	88.0	0.462
Negative	98	10	89.8	
TNM				
0–I	116	4	96.6	<0.001
IIa	173	20	88.4	
IIb	108	18	84.3	
III–IV	30	11	63.3	
Radiotherapy				
Yes	169	36	79.3	<0.001
No	224	14	93.8	
Chemotherapy				
Yes	419	50	88.3	0.042
No	17	5	70.6	

Table 2

Hazards ratios (HR) for overall survival (OS) and disease-free survival (DFS) in breast cancer patients by blood TGF- β levels

TGF- β 1 (ng/ml)	Total	No. death for OS/DFS	HR (95% CI) for OS/DFS	
			Univariate	Multivariate
Among all patients				
Q1 (0.130–4.764)	110	10/9	1.00/1.00 (reference)	1.00/1.00 (reference)
Q2 (4.764–7.468)	110	7/6	0.69(0.26–1.81)/0.66(0.24–1.86)	0.65(0.25–1.72)/0.60(0.21–1.76)
Q3 (7.468–11.082)	110	10/10	1.01(0.42–2.42)/1.11(0.45–2.74)	0.98(0.41–2.38)/1.07(0.43–2.65)
Q4 (11.082–37.99)	109	29/25	3.31(1.61–6.80)/3.14(1.46–6.72)	2.78(1.34–5.79)/2.49(1.15–5.41)
Among TNM-0, I, IIa patients				
Q1 (0.130–4.764)	86	7/6	1.00/1.00 (reference)	1.00/1.00 (reference)
Q2 (4.764–7.468)	71	2/2	0.34(0.07–1.62)/0.39(0.08–1.92)	0.38(0.08–1.82)/0.45(0.09–2.22)
Q3 (7.468–11.082)	72	3/3	0.50(0.13–1.94)/0.58(0.15–2.32)	0.51(0.13–2.00)/0.58(0.14–2.34)
Q4 (11.082–37.99)	60	12/8	2.65(1.04–6.73)/2.02(0.70–5.83)	2.47(0.96–6.35)/1.79(0.61–5.25)
Among TNM-IIb, In, IV patients				
Q1 (0.130–4.764)	22	3/3	1.00/1.00 (reference)	1.00/1.00 (reference)
Q2 (4.764–7.468)	37	5/4	0.99(0.24–4.16)/0.84(0.19–3.73)	1.01(0.24–4.23)/0.85(0.19–3.82)
Q3 (7.468–11.082)	36	6/6	1.27(0.32–5.08)/1.28(0.32–5.12)	1.55(0.38–6.29)/1.56(0.38–6.36)
Q4 (11.082–37.99)	43	15/15	3.03(0.88–10.46)/3.03(0.88–10.47)	3.16(0.91–10.9)/3.16(0.91–10.9)

Univariate and multivariate analysis (Adjusted for TNM staging, radiotherapy, and age at diagnosis, for all stages and for specified staging groups) are shown