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Role of TGF-β1 and its Receptors in Breast Carcinogenesis: Evaluation of Gene Expression Patterns and Clinical Implications

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OBJECTIVE Transforming growth factor β1 (TGF-β1) is a multifunctional cytokine that may play an important role in tumor development and progression.

METHODS We evaluated gene expression patterns of TGF-β1 and its receptors [transforming growth factor β type I receptor (TβR-Ⅰ) and transforming growth factor β type II receptor (TβR-Ⅱ)] in tumor tissue from patients with breast cancer or with benign breast diseases (BBD) and adjacent normal tissue from the patients with breast cancer. Included in the study were 527 breast cancer patients and 213 BBD patients who participated in the Shanghai Breast Cancer Study.

RESULTS The expression levels of the TGF-β1, TβR-Ⅰ and TβR-Ⅱ genes in breast tissue were quantified using real-time PCR. TβR-Ⅱ expression in cancer tissue was decreased by over 50% as compared to either adjacent normal tissue from the same patients or benign tumor tissue from BBD patients ($p<0.001$). TGF-β1 expression was lower by approximately 20% in cancer tissue compared to adjacent normal tissue ($p=0.14$) or to benign tumor tissue ($p=0.002$). Although TβR-Ⅰ expression was also reduced in cancer tissue compared to adjacent normal tissue, or benign tumor tissue, the magnitude of the reduction was less apparent than that for TβR-Ⅱ. Compared to patients with the lowest tertile value for TβR-Ⅱ, patients with median tertile value for TβR-Ⅱ had more favorable overall survival (HR 0.47, 95% CI 0.27-0.85) and disease-free survival (HR 0.65, 95% CI 0.39-1.06). No apparent associations, however, were observed between TGF-β1 or TβR-Ⅰ expression and overall or disease-free survival.

CONCLUSION The results from this study support the hypothesis that a decreased level of TβR-Ⅱ gene expression, and thus reduced TGF-β1 sensitivity, is related to breast tumor progression.

KEYWORDS: transforming growth factors, TGF-β, breast cancer, gene expression, survival.

INTRODUCTION

Transforming growth factor betas (TGF-βs) are multifunctional cytokines that regulate cellular division, differentiation, motility, adhesion, and death[1-4]. The functions of TGF-βs are mediated through their interaction with type Ⅰ and type Ⅱ TGF receptors (TβR-Ⅰ and TβR-Ⅱ). It has been well documented that TGF-βs are potent inhibitors of cell cycle progression in most normal epithelial cells[1-4]. Studies conducted in cell cultures and animal models have shown that TGF-βs inhibit the proliferation of tumor cells in the early stages of breast tumorigenesis[1-5]. In later stages of cancer, however, TGF-βs lose their inhibitory effect and act as tumor promoters[1-5]. It has been suggested that the loss of TβR-Ⅱ expres-

sion is related to resistance to the TGF-β-mediated inhibition of cell proliferation and tumor progression[1-5]. Reduced expression of TβR-Ⅱ and the resulting cellular resistance to TGF-βs have been reported in several cancer cell lines, including those from breast cancer [1-5].

Despite mounting evidence from in vitro experiments implicating an important role for TGF-βs in the pathogenesis of breast cancer, only a limited number of studies have been conducted directly using clinical samples obtained from patients with breast tumors[6]. It could be difficult to extrapolate data from in vitro studies directly to patients, as commonly-used cell lines were obtained from only a limited number of breast tumors, and cell culture media differ considerably from physiological conditions. Several previous studies have examined expression levels of TGF-βs and their receptors in human breast cancer tissue and non-neoplastic tissue[7-12]. The results from previous studies, however, have been inconsistent and sometimes even conflicting. There have also been several small studies relating evaluated breast cancer survival with the expression staus of TGF-βs and their receptors,[9, 10, 12-16] and again, the results were inconsistent. In this study, we systematically evaluated gene expression patterns of TGF-β1 and its receptors, TβR-Ⅰ and TβR-Ⅱ in breast tissue from patients with breast cancer or benign breast disease, and investigated whether the expression pattern of these genes may predict the prognosis in breast cancer patients.

MATERIALS AND METHODS

Subjects studied and tissue samples

Included in this study was a subset of patients who were diagnosed with breast cancer or benign breast disease between 1996 and 1998 who were recruited as part of the Shanghai Breast Cancer Study[17]. Patients studied were recruited through a rapid case ascertainment system that included 48 hospitals. These hospitals treated over 80% of the breast cancer patients in Shanghai. Breast tissue was collected from 543 cancer patients and 432 benign breast disease (BBD) patients. Most BBD patients were clinically suspected to have potential breast cancer. During the operation, tissue was removed from the center of the lesion as was another section (if available) from as far away as possible from the lesion. These samples were snap frozen in liquid nitrogen as soon as possible, typically within 10 minutes. Samples were stored at -70℃ until the relevant assays were performed.

All patients were interviewed at the time of recruitment. Medical charts were reviewed using a standard protocol to obtain information on cancer treatment, clinical stages and cancer characteristics, such as estrogen and progesterone receptor status. Two senior pathologists reviewed all tissue slides to confirm the diagnosis. Benign breast diseases were classified based on the published criteria developed by Page and colleagues[18].

As described previously[19], all breast cancer patients were followed through January 2003 via in-person or phone contacts and record linkage to the Shanghai Vital Statistics Unit. In all, 88.4% patients successfully completed the follow-up interview either in-person (85%) or by telephone (3.4%) between March 2000 and December 2002. For those who could not be contacted in person or by phone, linkage to the death certificate data was completed in June 2003 to obtain information on the date and cause of death. Subjects who had no match in the death registry were assumed to be alive on December 30, 2002, 6 months prior to the linkage, in order to allow for a possible delay of entry of the death certificates into the registry.

Laboratory assays

Total RNA was extracted from tissue specimens by homogenization in TRIzol solution, phase separation, precipitation and washing following the manufacturer's instructions (GibcoBRL, Carlsbad, CA). The quality and quantity of RNA were measured by spectrophotometric analysis. Reverse Transcription Reagents were obtained from Applied Biosystems (Foster, CA). RNA was reverse-transcribed in a final volume of 15 μl containing 0.15 μg RNA and 1× RT-PCR buffer, 5.5 mM $MgCl_2$, 500 μM each dNTP, 2.5 μM Random Hexamers, 0.4 U/μl Rnase inhibitor, and 3.125 U/μl MultiScribe reverse transcriptase (Applied Biosystems). The mixture was incubated at 25℃ for 10 min, 37℃ for 120 min and 95℃ for 5 min.

Quantitative real-time PCR was performed using a 384-well optic tray on a ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). A total reaction volume of 5 μl containing 2.2 μl cDNA template at different dilutions, 1×*Taq*Man Universal PCR Master Mix (without UNG), and 1× Gene expression Assay Mix including the primers, and marked probes from Applied Biosystems Assay-on-Demand services. The thermal cycling conditions were as follows: 95℃ for 10 min to activate the Ampli*Taq* Gold enzyme, followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min.

Every sample was tested in triplicate. Two control samples were used in each plate to monitor inter-plate variation, which was found to be smaller than 5% in our study. The threshold cycle (Ct) was determined at

0.1 based on the amplification linear area of the target genes and β-actin gene (an internal control). The normalized quantity of the target gene was calculated as $2^{-\triangle Ct}$, where $\triangle Ct$ was obtained directly by subtracting Ct for the target gene from Ct for the β-actin gene. The final result was expressed as $2^{-\triangle Ct}*1000$.

In total, there were tissues from 527 patients with primary breast cancer and from 213 patients with benign breast diseases. Also included in the study were paired adjacent normal tissues from 168 cancer patients. Because of assay failure for some samples, not all analysis included the complete set of the samples selected for the study.

Statistical analysis

The data were skewed to the high value, and thus geometric means were estimated and compared using the student's t-test or linear regression models. To evaluate associations of gene expression with breast cancer survival, patients were grouped based on the tertile distribution of the genes under evaluation, and the 5-year survival rate for each group was estimated using the Kaplan-Meier method. The log-rank test was applied to test the differences in survival across different genotypes. The Cox proportional hazard models were applied for calculating hazard ratios using the lowest tertile as a reference after adjusting for age and TNM stage. All p=values presented in this paper are two-sided. SAS software was used for statistical analysis (version 9.1; SAS Institute, Cary,NC).

RESULTS

The mean age was 46.8 years for the 527 breast cancer patients and 43.0 years for the 220 BBD patients ($p < 0.001$). Clinical characteristics of the patients with breast cancer and detailed histological categorization of the patients with BBD are provided in Table 1. Most patients had Stage Ⅱa (41%) or Stage Ⅱb (26%) cancer. Approximately 24% of patients were diagnosed at an early Stage (0 or Ⅰ), while 11% of the patients were diagnosed with Stage Ⅲ or Ⅳ cancer. Over 57% of the benign diseases were non-proliferative lesions. Among proliferative benign breast disease, fibroadenoma was the most common, accounting for 27.9% of the total benign disease diagnosed. Only four subjects (1.9%) had atypical hyperplasia, and they were combined with proliferative lesions in data analysis.

Presented in Table 2 are geometric means and 95% confidence intervals for the expression levels of the TGF-β1, TβR-Ⅰ, and TβR-Ⅱ genes in the tumor tissue of patients with cancer or BBD. TβR-Ⅱ expression was reduced in proliferative benign tumors as compared to non-proliferative tumors ($p = 0.077$), and further decreased in cancer tissue ($p < 0.001$). Cancer tissue showed an approximately 2-fold reduction in TβR-Ⅱ expression compared to benign tumor tissue, although, no apparent reduction was noted with the advance of clinical stage of the cancer. Similarly, expression of TβR-Ⅰ and TGF-β1 was decreased in

Table 1. Clinical characteristics of breast cancer and benign breast disease patients.

Item	No. of patients (%)
Breast cancer patients (by clinical stage)	
0 or Ⅰ	124 (24.0)
Ⅱa	210 (40.6)
Ⅱb	132 (25.5)
Ⅲ and Ⅳ	51 (10.9)
Benign breast disease patients (by histology*)	
Nonproliferative lesions	119 (57.2)
Cyst	2
Mild hyperplasia of the usual type	116
Mastitis, periductal mastitis	1
Proliferative lesions without atypia	85 (40.9)
Moderate floid ductal hyperplasia of the usual type	12
Intraductal papilloma	10
Sclerosing adenosis	5
Fibroadenoma	58
Atypical hyperplasia	4 (1.9)
Atypical ductal hyperplasia	4

*Based on the criteria of Dupont, Page, and Rogers[20].

Table 2. Geometric means (95% confidence intervals) of tumor tissue mRNA levels for the TGF-β1, TβR-Ⅰ, and TβR-Ⅱ genes in patients with breast cancer and benign breast disease.

Item	TGF-β type Ⅱ receptor	TGF-β type Ⅰ receptor	TGF-β Ⅰ	TGF-β Ⅰ /TβR-Ⅱ	TGF-β Ⅰ /TβR-Ⅰ
Benign breast diseases					
Nonproliferative lesions (n=124)	122.67 (107.13-140.47)	6.80 (6.06-7.63)	82.49 (67.74-100.46)	0.69 ((0.55-0.86)	11.13 (8.68-14.26)
Proliferative lesions (n=94)	102.21 (88.14-118.52)	7.56 (6.71-8.50)	73.04 (60.24-88.57)	0.70 (0.57-0.85)	9.18 (7.27-11.60)
P-value [a]	0.077	0.216	0.398	0.936	0.281
Breast cancer (by stage)					
0 and Ⅰ (n=124)	54.55 (46.83-63.53)	6.39 (5.81-7.03)	64.67 (58.06-72.03)	1.17 (1.00-1.36)	9.80 (8.42-11.41)
Ⅱa (n=210)	51.48 (45.46-58.30)	6.29 (5.76-6.87)	62.71 (57.63-68.23)	1.21 (1.08-1.36)	9.80 (8.63-11.12)
Ⅱb (n=132)	47.50 (40.52-55.69)	6.97 (6.17-7.88)	63.66 (56.49-71.74)	1.32 (1.12-1.55)	9.12 (7.84-10.61)
Ⅲ and Ⅳ (n=51)	70.61 (54.92-90.78)	6.21 (5.33-7.23)	70.24 (59.57-82.81)	0.99 (0.79-1.26)	11.31 (9.32-13.73)
P-value for trend test [b]	0.547	0.594	0.587	0.858	0.757
Benign breast diseases (n=218)	113.18 (102.35-125.15)	7.13 (6.56-7.74)	78.26 (68.08-89.96)	0.69 (0.59-0.81)	10.22 (8.60-12.15)
Breast cancer (n=517)	52.73 (48.73-57.05)	6.48 (6.13-6.84)	64.12 (60.69-67.75)	1.20 (1.11-1.30)	9.76 (9.04-10.53)
P-value [c]	<0.001	<0.001	0.002	<0.001	0.577

a Comparing by BBD histological classification.
b Comparing by breast cancer stages assuming a linear trend.
c Comparing BBD and cancer patients.

cancer tissue compared to benign tumor tissue, but no further reduction was seen for advanced cancer. A measure of the sensitivity of TGF-β1 toward its receptors, the TGF-β1/ TβR-Ⅱ ratio, was significantly higher for cancer tissue than for benign tumor tissue ($p < 0.001$). On the other hand, the TGF-β1/ TβR-Ⅰ ratio did not differ among cancer and benign tumor tissues.

Table 3 evaluates the expression levels of the TGF-β1, TβR-Ⅰ, and TβR-Ⅱ genes in cancer tissue and paired adjacent normal tissue from breast cancer patients. TβR-Ⅱ gene expression was lowered by about 60% in cancer tissue compared to adjacent normal tissue ($p<0.001$). The expression of the TGF-β1gene was also reduced in cancer tissue, although the reduction was not statistically significant. Overall, cancer tissue showed a 90% increase in the TGF-β1/ TβR-Ⅱ ratio over adjacent normal tissue ($p < 0.001$). TβR-Ⅰ expression was only slightly decreased in cancer tissue, and the TGF-β1 to TβR-Ⅰ ratio was similar between cancer tissues and adjacent normal tissues.

Table 4 provides the association of TβR-Ⅱ and TGF-β1 expression levels in cancer tissue with breast cancer patient survival. The median follow-up time for breast cancer patients was 5.1 years. Compared to patients in the lowest tertile of TβR-Ⅱ expression, patients in the median tertile had more favorable overall survival (HR 0.47, 95% CI 0.27-0.85) and disease-free survival (HR 0.65, 95% CI 0.39-1.06), whereas survival for patients in the highest tertile was similar to that of patients in the lowest tertile. A similar pattern of association was observed for the TGF-β1/ TβR-Ⅱ ratio.

Table 3. Geometric means (95% confidence intervals) of mRNA levels for the the TGF-β1, TβR-Ⅰ, and TβR-Ⅱ genes in cancer and adjacent normal tissues from patients with breast cancer.

Item	Tumor tissue	Adjacent normal tissue	Percent difference[a]	p-value[b]
TGF-β type Ⅱ receptor (n=140)	52.09 (45.00-60.30)	121.22 (103.31-142.22)	-57.0	<0.001
TGF-β type Ⅰ receptor (n=112)	6.50 (5.81-7.26)	7.20 (6.48-7.99)	-9.7	0.055
TGF-βⅠ (n=133)	70.87 (63.68-78.86)	85.09 (75.68-95.67)	-16.7	0.142
TGF-βⅠ/ TβR-Ⅱ (n=112)	1.38 (1.19-1.61)	0.73 (0.62-0.87)	89.0	<0.001
TGF-βⅠ/ TβR-Ⅰ (n=98)	10.90 (9.32-12.73)	10.73 (9.20-12.51)	1.6	0.853

[a]Calculated by [(tumor tissue-adjacent normal tissue)/adjacent normal tissue]×100.
[b]Derived from paired t-test.

Table 4. Association of tumor tissue mRNA levels of the TβR-Ⅱ and TGF-β1 genes with breast cancer patient survival.

	Overall survival					Disease-free survival			
	No. of patients	No. of event	5-year survival rate (%)	Unadjusted HR (95% CI)	Adjusted HR[a] (95% CI)	No. of event	5-year survival rate (%)	Unadjusted HR (95% CI)	Adjusted HR[a] (95% CI)
TGF-β type Ⅱ receptor									
T1	168	36	79.9	1.0	1.0	40	76.3	1.0	1.0
T2	171	17	90.3	0.43 (0.24-0.77)	0.47 (0.27-0.85)	26	84.4	0.59 (0.36-0.97)	0.65 (0.39-1.06)
T3	168	32	81.0	0.86 (0.54-1.39)	0.94 (0.58-1.51)	37	78.3	0.89 (0.57-1.40)	0.98 (0.63-1.53)
TGF-β1									
T1	175	32	82.4	1.0	1.0	38	78.0	1.0	1.0
T2	173	25	87.1	0.76 (0.45-1.28)	0.76 (0.45-1.28)	33	81.6	0.86 (0.54-1.38)	0.86 (0.54-1.37)
T3	174	27	83.8	0.83 (0.50-1.38)	0.83 (0.49-1.38)	34	80.1	0.88 (0.55-1.39)	0.88 (0.55-1.39)
TGF-β1/ TβR-Ⅱ ratio									
T1	169	30	82.4	1.0	1.0	38	77.9	1.0	1.0
T2	168	20	89.4	0.64 (0.37-1.14)	0.59 (0.33-1.04)	23	86.0	0.57 (0.34-0.96)	0.52 (0.31-0.88)
T3	168	34	80.0	1.19 (0.73-1.95)	1.10 (0.67-1.80)	41	75.6	1.13 (0.73-1.75)	1.05 (0.67-1.63)

[a]Adjusted for age and stage.

DISCUSSION

In this study we found that expression levels of the TβR-II gene were substantially reduced with the progression from benign breast disease to cancer. The reduction was noted as early as in proliferative BBD, and was particularly evident in cancer tissue. As a result of the reduced expression of the TβR-II gene, the ratio of TGF-β1 to TβR-II, as a measure of TβR-II sensitivity, was increased in the development of breast cancer. These results suggest strongly that loss of TβR-II expression and subsequent resistance to the TβR-II-mediated growth inhibitory pathway plays a significant role in the development of breast cancer.

Our results are supported by data from in vitro and in vivo studies implicating an important role of the TGF-β type II receptor in breast carcinogenesis. It has been reported that the levels of expression and responsiveness are closely correlated,[1-5] and the restoration of leads to the reversion of malignancy in human breast cancer cell lines[1-5]. Animal studies have shown that mice conditionally knocked out for the TβR-II gene in the mammary epithelium show lobular –alveolar hyperplasia in the developing mammary gland, and have shortened median tumor latency and an increased formation of pulmonary metastases [22]. In agreement with our results, Gobbi et al.[11] reported that neoplastic cells from cancer patients showed reduced TβR-II expression in comparison to normal breast tissue and benign breast lesions. Only one study to date has compared TβR-II expression in breast cancer and adjacent normal tissues[12]. Immunohistochemistry was used in that study, and the percentage of expression was found to be similar between cancer tumor and adjacent normal tissues.

We also observed that the expression of the TGF-β1and TβR-I genes in cancer tissues was reduced compared to benign tumor tissue and adjacent normal tissue. The differences, however, were substantially smaller than that of the TβR-II gene. Similarly, only a few previous studies have compared TGF-β1and TβR-I expression in tissues from cancer patients and benign disease patients or adjacent normal tissue[7,9,10,12]. Our results were inconsistent from that reported by Buck et al.[12], in which a significantly higher proportion of positive TβR-I expression was found in cancer tissue as compared to normal tissue. Again, in contrast to our findings, several previous studies have shown that breast cancer tissue had a higher level of TGF-β expression than non-malignant breast tissue[7,9,10].

The reasons for the inconsistent findings are unlear. Most previous studies employed a very small sample size. In our study mRNA levels of TGF-β1, TβR-I and TβR-II were quantified, whereas in previous studies, protein levels were measured using semi-quantitative techniques such as immunohistochemistry or Northern blots. Furthermore, TGF-β1 and its receptors in our study were measured in terms of relative amounts to the house-keeping genes.

We found in our study a substantially reduced mortality rate among breast cancer patients whose cancer tissue expressed a moderate amount of TβR-II mRNA. The risk, however, was similar among women with the lowest or the highest expression of this gene in their breast cancer tissue. We have no satisfactory explanation for this U-shape association. Given the dual role of TGF-β1 in breast carcinogenesis,[4] the U-shape association may be biologically plausible. Only one study has reported on the association of breast cancer survival with TβR-II expression. In that study, immunohistochemistry was used, and TβR-II expression was associated with poorer overall survival, especially in estrogen receptor-negative patients[12]. These results were contradictory to ours. For TGF-βs and breast cancer patient survival, the results were again inconsistent. We found that a high TGF-β1 expression was related to a favorable prognosis, although the association was not statistically significant. Similar associations were reported from several previous studies[9, 10, 13, 16]. On the other hand, one study has reported no association[15].

The large sample size of the study provides stable estimates of gene expression patterns and their correlation with cancer patient survival. In addition, we included both tumor and adjacent normal tissues of breast cancer and tumor tissue of benign breast diseases, which enables a systematic evaluation of the expression TGF-β levels of genes according to breast tumor progression. Although RT-PCR allows us to quantify the expression of target genes, this technique only measures steady mRNA levels. It may be a concern if mRNA levels do not reflect levels of the protein itself[21]. However, TGF-β1 mRNA was found only in tumor tissues with detectable TGF-β1 proteins, and TGF-β1 mRNA expression has been correlated with TGF-β1 protein in a series of breast carcinomas[22].

In conclusion, our data provide evidence that resistance to the TβR-II-mediated growth inhibitory effect occurs during breast cancer carcinogenesis, and that TβR-II gene expression in cancer tissue is related to survival of breast cancer patients. The gene expression level of TβR-II may be a useful predictor of breast tumor aggressiveness.

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