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Cessation of Dormancy Involves an Increase of the Expression of TGFβ1 at Protein Level in Breast Cancer

Kristiina Joensuu[1*], Marja Heiskala[1], Päivi Heikkilä[1], Marjut Leidenius[2]

[1]Department of Pathology and HUSLAB, Helsinki University Hospital and University of Helsinki, FIN-00290 Helsinki, Finland

[2]Department of Breast Surgery, Comprehensive Cancer Center Helsinki University Hospital, P.O Box 163, 00290 HUS, Helsinki, Finland University of Helsinki, Central Hospital, FIN-00290 Helsinki, Finland

[*]**Corresponding author:** Kristiina Joensuu, Department of Pathology and HUSLAB, Helsinki University Hospital and University of Helsinki, FIN-00290 Helsinki, Finland

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Abstract

The duration of dormancy of breast cancer after removing the primary tumor is unpredictable, and depends on the properties of the malignant epithelial cells and on the immunological response of the host. In this study, we investigated the role of TGFβ signalling in tumor progression by estimating the expression TGFβ1, TGFβ2 and TGFβ3 at protein level in primary tumours and the corresponding recurrences. The frequency of Foxp3 transcription factor positive T cells was also evaluated. We used 137 paraffin fixed samples of primary breast cancer and their corresponding recurrent lesions, first recorded 0-2, 5-10 and >10 years after the diagnosis. The results showed that the expression of TGFβ1was significantly higher in the recurrent lesions than in the primary tumors, regardless of tumor type and state. The frequency of intratumoral Foxp3 positive lymphocytes was associated with the tumor cell expression of TGFβ1 in the primary tumors. The protein level expression of none of the TGFβ isoforms nor Foxp3 predicted the duration of the dormancy. In primary tumors TGFβ1 associated with lobular histology, ER and PR positivity and a low grade, TGFβ2 with a smaller tumor size, and TGFβ3 with ductal type of the tumor, a high expression of Ki67 and HER2 positivity.

Keywords: Breast cancer dormancy; TGFβ-signalling

Introduction

Tumor dormancy after surgical treatment and individually designed adjuvant therapy of breast cancer depends on interactions between the remaining malignant cells and the immune response of the host. We have previously shown that a high expression of AZIN1 in tumor cells as well as in the microenvironment [1], and an activation of the CCL2 pathway in the tumor stroma [2], predict an early recurrence. In order to further investigate the transition of early (primary) breast cancer into a disseminated disease, we examined the protein level expression of TGFβ1, 2 and 3, and the frequency of Foxp3 positive T cells in primary tumors recurring at three different time points (early, intermittent, late), and in their corresponding recurrent or metastatic lesions.

TGFβ belongs to a large family of proteins with activity on cell growth and differentation, including the activin/inhibin subfamily, the Bone Morphogenetic Proteins (BMPs), nodal, myostatin, and the Mullerian Inhibitory Substance (MIS) [3-6]. Three distinct isoforms of TGFβ have been identified in mammalian cells (TGFβ-1, -2, -3), each encoded by a different gene [7-10]. Cell function regulation by TGFβ is conducted via an interaction with cell surface receptors I, II, III (TβRI, II, III) [11-14]. Both TGFβ and its receptors are widely expressed in all cell types, and defects in TGFβ signaling are associated with various human diseases from autoimmunity to cancer. The active TGFβ molecule, synthesized from a large inactive precursor molecule, the latent TGFβ, is a homodimer stabilized by hydrophobic interactions strengthened by a disulfide bond [15].

The role of TGFβ signaling in controlling the life of normal and malignant cells is dual: in normal cells and early cancer it inhibits cell proliferation and immortalization and induces apoptosis, whereas in aggressive cancers the signaling via TGFβRII becomes defective because of genetic and epigenetic modifications in the TGFβ signaling components, or because cells become resistant to the signaling, due to the activation of pro-oncogenic signaling pathways (MAPK, PI3 K, Ras, c-MYC), which override the growth inhibitory signaling pathways, including TGFβ/Smad [16-19]. The tumor suppressive functions are then replaced by induction of Epithelial-Mesenchymal Transition

(EMT), loss of cell adhesion, increased migration, invasion, chemo attraction, and tumor metastasis [20-25]. An important part of the pro-tumoral effects of the TGFβ pathway consists of the capability to mediate and modify tumor-stroma interactions and to remodel tumor microenvironment [26]. The role of TGFβ signaling in the induction of Foxp3+ regulatory T cells has recently been intensively investigated, and simultaneously a direct, Foxp3-independent TGFβ-mediated function regulating auto reactive T cells and maintaining peripheral T cell tolerance has also been recognised [27].

The impact of TGFβ signalling has primarily been evaluated based on observations on the TGFβ1 isoform, whereas few articles describe the potentially different roles of the other TGFβ isoforms. The isoforms seem to have different functions during mammary gland development and involution [28-31], and different patterns of expression have also been reported in different types of cancer. A higher expression of TGFβ1 and TGFβ3 has been reported in breast cancer versus normal tissue, with no difference in the expression of TGFβ2 [32,33]. TGF isoforms have also been reported to have distinct patterns of co-expression with TGFβ receptors, depending on the type of cancer.

In this study we evaluated the role of TGFβ signaling in tumor dormancy and progression in breast cancer by investigating the expression of TGFβ1, TGFβ2 and TGFβ3 at protein level in the primary tumors with different prognoses. We also compared their expression in primary tumors versus corresponding recurrent or metastatic lesions. To follow the possible immunomodulating impact of the TGFβ pathway, we investigated the frequency of Foxp3+ T cells in the same set of samples.

Materials and Methods

Patients and tissue samples

Paraffin-embedded tissue blocks from Primary Breast Tumors (PTs) of 137 patients and their corresponding Recurrent Lesions (Rs) were collected from the archives of the Department of Pathology, Helsinki University Hospital as described previously [34]. The primary tumors had been removed in 1974-2006. The cases were selected based on the time lapse from the primary operation to the first recurrence to represent quickly (≥2 years), intermediately (5-10 years) and slowly (>10 years) progressing tumors. All consecutive cases fulfilling the requirement of the treatment-relapse interval were recruited, and three groups were formed: Group 1 n=41, tumors with relapse (R) within two years after primary surgery, Group 2 n= 57, with R after 5 -10 years and Group 3 n=39 with R after > 10 years (range 10 to 23 years). The archival slides were re-examined, and the histological tumour type and grade were assigned based on the criteria of Elston and Ellis [35]. The clinico pathologic characteristics of the patients and their cancers are summarized in Table 1. The Ethical Committee of the Helsinki University Central Hospital approved the study protocol.

	Group 1 n=41	**Group 2 n=57**	**Group 3 n=39**
Age at surgery of primary tumor (PT)			
< 50 years	19	20	18
≥ 50 years	22	37	21
Tumor size			
≥ 20 mm	14	28	24
< 20 mm	26	28	15
system missing	1	1	
Lymph node			
negative	14	34	21
positive	24	20	13
system missing	3	3	5
Grade			
1	4	7	8
2	22	35	26
3	15	15	5

Histological type			
ductal	24	36	16
lobular	17	19	23
special types	0	2	0
Tissue site of recurrence (R)			
skin	6	10	11
soft tissue	6	12	5
subcutaneous tissue	12	16	15
lung	0	4	2
brain	2	2	0
lymph node	2	1	2
ovary	0	1	0
bone	3	6	4
liver	5	2	0
pleura	0	1	0
peritoneum	2	1	0
mesenterium	1	0	1
larynx	1	0	0
uterus	1	0	1
duodenum	0	1	0

NOTE. In Group 1 recurrences were detected within two years, in Group 2 from 5 to 10 years and in Group 3 >10 years after primary surgery. R was defined as any local or regional recurrence or any distant metastatic disease. Adapted from ref. 2 where the same set of tumor samples was used.

Table 1: Clinicopathologic parameters of the 137 breast cancer patients and the site of recurrence.

Immunohistochemistry

The protein level expression of the three human isoforms of the cytokine TGFβ, TGFβ1, TGFβ2, TGFβ3, and the transcription factor Fork Head Box P3 (Foxp3) was investigated in the samples, and the grading of the expression level was based on viewing the entire tumor sections at the 20x magnification. The type of the marker-positive cells was specified using the 40x magnification. For TGFβ1, TGFβ2 and TGFβ3 the expression level in tumor cells was scored as 0: no antigen expression, +1: 5-29% of antigen expressing cells, +2: 30-69% of antigen expressing cells, and +3: 70-100% of antigen expressing cells. The evaluation of the frequency of Foxp3 expressing lymphocytes was performed according to Sobottka et al. [36], with tuFoxp3 representing intratumoral Foxp3 positive lymphocytes in direct contact with tumor cells, and strFoxp3 representing Foxp3 positive lymphocytes within stromal areas of the invasive tumor. The frequency was scored as negative: no Foxp3 expressing lymphocytes, +1: 5-29% of lymphocytes expressing Foxp3, +2: 30-69% of lymphocytes expressing Foxp3, and +3: 70-100% of lymphocytes expressing Foxp3.

The staining results for ER, PR, HER2 and MIB1 was evaluated according to a standard protocol [37,38]. All tumors with an over expression of HER2 at protein level [34,38] were tested for HER2 gene amplification by Inform HER2 Dual ISH test (Inform HER2 Dual In Situ Hybridization) [39].

Four μm thick sections were deparaffinised in xylene and rehydrated. To block endogenous peroxidase, the slides were treated in a PT module (LabVision UK Ltd.Suffolk, UK) in Tris-HCL buffer (ph 8,5) for 20 min at 98°C and with 0,3% Dako REAL Peroxidase Blocking Solution for 15 min. Immunostaining was performed in an Auto stainer 480 (Lab Vision Thermo scientific, UK Ltd.Cheshire, UK) by addition of the primary antibodies, see

below, followed by 30 min incubation with Dako RealEnVision/HRP detection system, Rabbit/Mouse (ENV) reagent (Dako, K5007), and the visualization of staining was done by REAL DAB+Chromogen (Dako, K5007) for 10 min. Washing with PBS-0,04%-Tween20 took place between each step. Both staining's were counterstained with Mayer`s hematoxylin and mounted in mounting medium.

As primary antibodies the following reagents were used: polyclonal rabbit anti TGFβ1, anti TGFβ2, and anti TGFβ3 antibodies at dilution 1:200, 1:500 and 1:500, respectively, (Spring Bioscience USA), mouse monoclonal anti Foxp3 clone 236A/E7 at dilution 1:500, (Abcam United Kingdom), mouse monoclonal anti-ER alfa clone 6F11, at dilution 1:50 (Novo Castra Newcastle United Kingdom), anti-PR alfa clone 636, at dilution 1:100 (DacoCytomation Denmark), anti-HER2 clone CB11, at dilution 1:400 (Novo Castra United Kingdom), and anti-Ki67 clone MIB-1, dilution 1:75 (Daco Cytomation Denmark).

Normal human placenta tissue was used as positive control for TGFβ1, TGFβ2 and TGFβ3, and normal human tonsil tissue for Foxp3.

The samples were analysed independently by three different pathologists (KJ, MH, PH) for scoring, and a partial re-evaluation confirmed the reproducibility of the results.

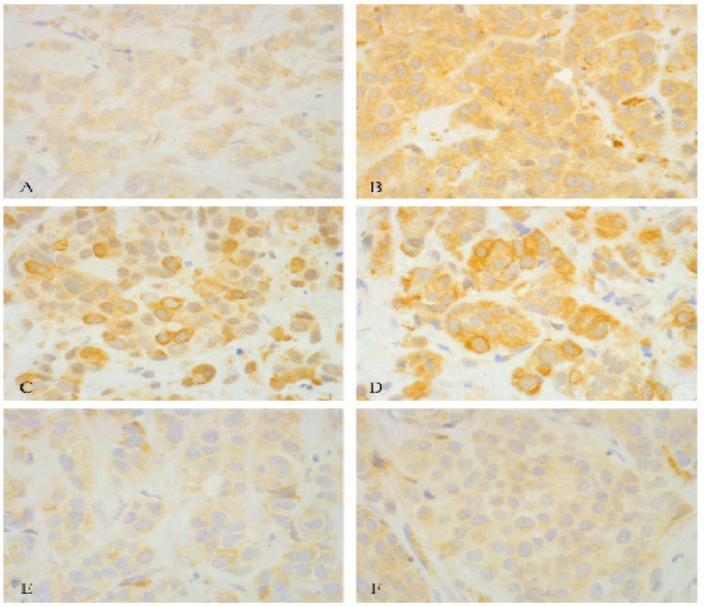
Statistical methods

All statistical analyses were performed using SPSS 24.0 for Windows (SPSS Incorporation, Chicago, IL, USA). The differences between the expression of the markers in PTs and the corresponding Rs within the Groups were tested using the paired samples t-test. Kruskal-Wallis test and the Mann-Whitney U test was used for comparing differences between the Groups. For analyzing the association of the expression of the markers with the Clinicopathologic parameters, ER, PR, Ki67 and HER2 we used the categorical two-tailed Pearson's chi-square test. For Pearson's Chi-square tests, the cut-off point for negativity versus positivity for TGFβ1, TGFβ2 and TGFβ3 was 0 versus ≥1, and for Foxp3 < 2 versus ≥ 2. For ER and PR the cut-off point for positivity was 1%, and for Ki67 ≥14%. Regarding HER2, only the tumors with a positive gene amplification were considered HER2 positive. Probability values $p<0.05$ were considered significant except in the Mann-Whitney U test, where $P < 0,0167$ (< 0,5/3) was used.

Results

TGFβ1

In the entire tumor set TGFβ1 was widely expressed; the frequency of TGFβ1 positive tumor cells was ≥1 in 59 (43,1%) of the Pts and in 111 (81,0%) of the Rs. The TGFβ1 positivity was high (≥2+) in 10 (7,3%) cases of the Pts and in 26 (19%) of the Rs. The protein was evenly distributed in the cytoplasm and nucleus (Figure 1).



A. TGFβ1 in Pt, B. TGFβ1 in R, C. TGFβ2 in Pt, D. TGFβ2 in R E. TGFβ3 in Pt, F. TGFβ3 in F

Figure 1: Expression of TGFβ1, TGFβ2 and TGFβ3 in a sample of Pt of breast cancer and in its corresponding R lesion after 9 years; immunohistochemical staining at 40x magnification.

The mean expression TGFβ1 at protein level was significantly higher in the Rs as compared to the corresponding Pts in the whole set of samples (p=0.0001). The difference was significant also in all the three Groups analysed separately; the p-value was 0.001 in Group 1, 0.0001 in Group 2 and 0.001 in Group 3 (Figure 2). When compared to each other, the Groups showed the same level of TGFβ1 expression; time to relapse was not reflected in the expression in the Pts nor in the corresponding Rs (Figure 2).

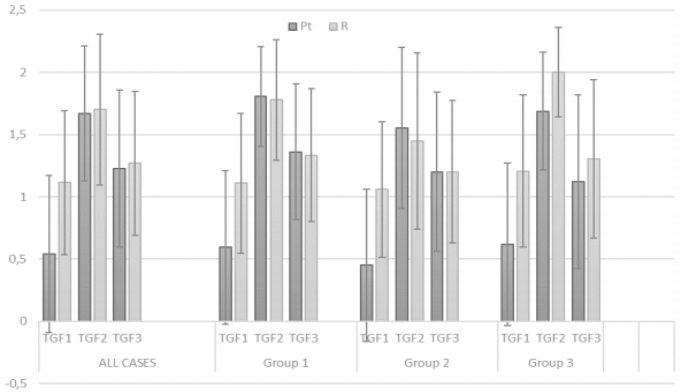


Figure 2: Mean expression levels of TGFβ1, TGFβ2 and TGFβ3 in primary tumors and corresponding recurrent lesions in the entire set of 137 pairs of samples, and separately analysed in Groups 1, 2 and 3 with short, intermediate and long time, respectively, to progression. The expression levels were determined by IHC.

The expression of TGFβ1 (> 0) correlated positively with the expression of ER (p=0.074), and PR (p=0.055), although the result did not reach statistical significance. The other conventional prognostic markers of breast cancer, Ki67 and HER2, did not show correlation. The expression of TGFβ1 was more often negative in ductal than in lobular breast cancer (p=0.046), and in high grade (2-3) than in low-grade (1) tumors (p=0.014). TGFβ1 positivity versus negativity did not correlate with the nodal status (Table 2).

	PR negative	**PR positive**	***p***
TGFβ 1 negative	25 (37,9%)	41 (62,1%)	
TGFβ 1positive	13 (22,0%)	46 (78,0%)	0,055
	ductal type	lobular type	
TGFβ 1 negative	42 (62,7%)	25 (37,3%)	
TGFβ 1positive	26 (44,8%)	32 (55,2%)	0,046
	low grade	high grade	
TGFβ 1 negative	4 (5,9%)	64 (94,1%)	
TGFβ 1 positive	12 (20,3%)	47 (79,7%)	0,014
	FoxP3 low	FoxP3 high	
TGFβ 1 negative	56 (86,2%)	9 (13,8%	
TGFβ 1 positive	30 (52,6%)	27 (47,4%)	0,0001
	size < 20 mm	size ≥20 mm	
TGFβ 2 negative	3 (100%)	0 (0%)	
TGFβ 2 positive	51 (43,6%)	66 (56,4%)	0,052
	Ki67 negative, <14%	Ki67 positive, ≥14%	
TGFβ 3 negative	11 (84,6%)	2 (15,4%)	
TGFβ 3 positive	56 (52,8%)	50 (47,2%)	0,029
	ducatal type	lobular type	
iFoxP3 low	54 (62,8%)	32 (37,2%)	
iFoxP3 high	11 (31,4%)	24 (68,6%)	0,002
	low grade	high grade	
iFoxP3 low	7 (8,0%)	80 (92,0%)	
iFoxP3 high	8 (22,2%)	28 (77,8%)	0,029
	ER negative	ER positive	
iFoxP3 low	37 (43,0%)	49 (57,0%)	
iFoxP3 high	6 (17,1%)	29 (82,9%)	0, 007
	size < 20 mm	size ≥20 mm	

sFoxP3 low	48 (50,0%)	48 (50,0%)	
sFoxP3 high	3 (18,8%)	13 (81,3%)	0,020
	ductal type	lobular type	
sFoxP3 low	48 (50,5%)	47 (49,5%)	
sFoxP3 high	13 (81,3%)	3 (18,8%)	0,022
	Ki67 negative, <14%	Ki67 positive, ≥14%	
sFoxP3 low	59 (62,8%)	35 (37,2%)	
sFoxP3 high	4 (25,0%)	12 (75,0%)	0,005

Table 2: Relationship between TGFβ1, TGFβ2, TGFβ3 and Foxp3 with ER, PR, Ki67 IHC expression and HER2 gene amplification in the 137 primary tumors of breast cancer. The categorical Pearson's chi-squared test ($\chi 2$) was used. Only significant results are shown.

TGFβ2

TGFβ2 was widely expressed in the entire tumor set; the frequency of TGFβ2 positive tumor cells was ≥1 in 119 (86,9%) of the Pts and 117 (85,4%) of the Rs. The expression in tumor cells was high (≥2+) in 85 (62,0%) cases of PTs and 92 (67,2%) in Rs. Notably, the expression of TGFβ2 in the epithelial tumor cells was higher than that of TGF-β1 and TGFβ3, and the subcellular distribution of the protein was typical for TGFβ2, the samples showing granular cytoplasmic and frequently prominent cell membrane-associated staining (Figure 1 and 2).

The expression of TGFβ2 did not significantly vary between the PTs and the Rs when looking at the whole set of samples. When analysing the Groups separately, a higher expression in Rs than in the corresponding Pts was shown in Group 3 (p=0.010).

There was a significant difference in TGFβ2 mean expression between the Rs of the three Groups (p=0.0001, Kruskal-Wallis test, data not shown). Analysed by the Mann-Whitney test the difference was significant between the Groups 2 and 3, the expression being higher in the Group 3 (p=0,0001 (<0,167, <0,5/3), data not shown).

A high expression of TGFβ2 associated with the lobular histological type of the tumor (p=0,018), and TGFβ2 negative tumors were larger (>20 mm) than those showing TGFβ2 expression (p=0.052), but no correlation was shown to the other conventional prognostic markers of breast cancer, nor to the stage of the tumor (Table 2).

TGFβ3

In the entire tumor set TGFβ3 was widely expressed; the frequency of TGFβ3 positive cells was ≥1 in 109 (79,6%) of the Pts and in 117 (85,4%) in the Rs. The expression was high (≥2) in 40 (29,2%) PTs and 43 (31,4%) in Rs. The protein was evenly distributed in the cytoplasm and nucleus (Figure 1).

The expression levels of TGFβ3 did not differ significantly between the PTs and the Rs in the entire tumor set nor in the Groups analysed separately. There were no significant differences in the expression of TGFβ3 between the Groups, neither with respect to the PTs nor the Rs.

A high expression of TGFβ3 tended to associate with HER2 over expression (p=0.056), and TGFβ3 negativity associated with a low expression of Ki67 (p=0.029), but no correlation was shown to other conventional prognostic markers of breast cancer, nor to the stage of the tumor (Table 2).

Foxp3

In the entire set of samples Foxp3 positive lymphocytes infiltrated the tumors in 87 (63, 5%) of the PTs and in 94 (68, 6%) of the Rs, and the stroma in 97 (70, 8%) of the PTs and in 93 (67, 9%) of the Rs. A high frequency (≥2) of Foxp3 positive lymphocytes was seen intratumorally in 36 (26, 3%) of the Pts and in 28 (20, 4%) of the Rs, and stromally in 16 (11, 7%) of the Pts and in 18 (13, 1%) of the Rs. The frequency of Foxp3 positive lymphocytes was similar in Pts and in their corresponding Rs both in the entire set of samples, and in the Groups analysed separately (Figure 3).

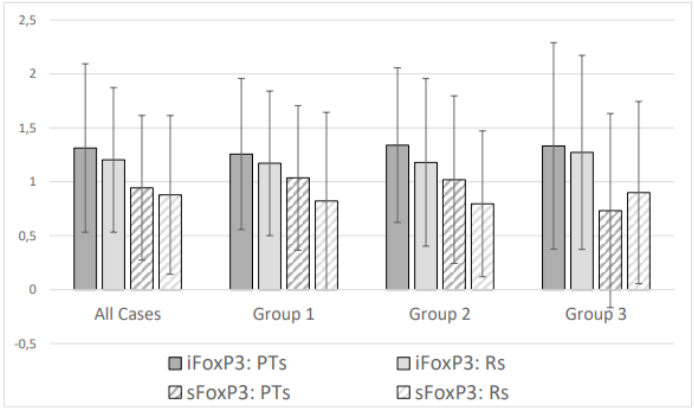


Figure 3: Mean frequencies of Foxp3 positive intratumoral and stromal lymphocytes in primary tumors and corresponding recurrent lesions in the entire set of 137 pairs of samples, and separately analysed in Groups 1, 2 and 3 with short, intermediate and long time, respectively, to progression.

A high expression of intratumoral Foxp3 tended to be associated with ER and PR positivity (p-values 0.007 and 0.068, respectively) and with the lobular histology of the tumor (p=0.002). A low expression of intratumoral Foxp3 associated with TGFβ1 negativity, and with a high (2-3) tumor grade (p=0,029) (Table 2).

A high expression of stromal Foxp3 (strFoxp3) associated with a high Ki67 positivity (p=0,005), a larger tumor size (< 20mm, p=0,020) and with the ductal type of the tumor (p=0,022) (Table 2).

Discussion

The impact of the TGFβ signaling in cancer has primarily been investigated based on observations on TGFβ1, whereas the role of the other TGFβ isoforms remains uncertain. The three TGFβ isomers have been shown to have different functions during mammary gland development and involution [28-31], suggesting that their impact on tumor development is different as well. In this study our aim was to determine the correlation of the protein level expression of the three TGFβ isoforms and Foxp3 with the duration of dormancy after the treatment of primary breast cancer, and to compare the expression levels in primary tumors versus the corresponding recurrent and metastatic lesions to evaluate their role in tumor progression.

TGFβ1

Our results indicated that immunoreactive TGFβ1 was found in the majority of the samples from primary invasive breast cancer, and the expression level was significantly higher in the corresponding metastatic lesions. We found no significant correlation between the mean levels of TGFβ1 protein expression and ER, PR, HER2, histology, aggressiveness nor nodal status, whereas when comparing TGFβ1 negative tumors (0) versus those expressing TGFβ1 (≥1) we saw TGFβ1 to be associated with some of the favorable prognostic markers (ER and PR expression, low grade of tumor). This is in agreement with the current consensus of TGFβ protein expression correlating in early-stage tumors with a favorable prognosis, and in advanced tumors with tumor aggressiveness and poor prognosis [32,40-44]. We saw a uniform cytoplasmic staining in cancer cells, whereas previously, secreted TGFβ1 has been shown to strongly localize to the advancing edge of the tumor [44]. The difference in choosing epitopes for immunization to produce antibodies against TGFβ1 may explain the diversity of the results. In our study TGFβ1 negative tumors were more of the ductal than lobular histology, the latter known to have a less favorable prognosis [45]. This finding might reflect a different mechanism of tumor induction in ductal versus lobular breast cancer. Moreover, while tumor type-dependent differences in the mRNA level expression of TGFβ1 have previously been reported, a clear non-accordance between TGFβ1 mRNA and protein levels has been noted, too, and this makes the interpretation of the findings complicated and method-dependent [33,46]. In our material the level of the TGFβ1 protein expression in primary tumors did not differ between the Groups with different times to progression, meaning that it had no prognostic value. Understandably the complexity of activating the latent TGFβ1 protein, and the dual role of TGFβ1 in the progression of cancer make the mere presence of immunoreactive TGFβ1 an insufficient marker to monitor the biological behavior of the tumor.

TGFβ2

In our study TGFβ2 was widely expressed both in primary breast cancer and in recurrences. The signal for the protein was over all stronger than that of TGFβ1 and TGFβ3. The expression level was similar in Pts in all the Groups, indicating that time to progression had no impact. Pts and Rs did not differ from each other except for the Group 3, where the expression was significantly higher in Rs than in Pts. In Rs, the expression tended to be higher in the Groups with a long dormancy. In Group 3 it was significantly higher than in Group 2. We also noted an association of a high expression of TGFβ2 with lobular histology of breast cancer.

Higher levels of TGFβ1 and TGFβ3 mRNA expression have been shown in cancer patients compared to normal tissues, and in more advanced tumors compared to early stage tumors, with no significant changes in TGFβ2 expression [32,33]. High TGFβ2 mRNA levels in breast tumours have been reported to predict a better better prognosis especially in lymph node-negative diseases [32]. However, an up-regulated TGFβ2 mRNA level in advanced compared to early tumors has also been reported, and an inverse correlation between TGFβ2 protein and mRNA has been

documented [47]. Our observation of a tendency to an increase of TGFβ2 at protein level in metastatic lesions in late recurring breast cancer is new, and suggests an active role for TGFβ2 in the process of the termination of tumor dormancy. Lobular histology is associated with a prognosis inferior to ductal [45], and our observation of a high TGFβ2 expression at protein level in tumors with this histology may have a causal connection. Malignant transformation and progression may differ between the histological subtypes of breast cancer, and may utilize different parts of the TGFβ pathway.

TGFβ3

In our study the expression levels of TGFβ3 did not differ significantly between the Pts and the Rs in the entire tumor set nor in the Groups analysed separately, and the time to progression had no impact on the expression in Pts nor in Rs.

TGFβ3 has an isoform-specific function in embryonic palate fusion and wound healing [48-50], but its possible role in tumorigenesis and tumor progression has only sporadically been studied. A high expression of TGFβ3 at mRNA level has been noted in breast tumors with a high stage, the protein level, however, remaining elusive (32). Elevated levels of TGFβ3 expression have been reported in late-stage tumours [51], and in glioblastomas a high expression of TGFβ3 has been reported to correlate with a poor prognosis [52]. An experimental study in these tumors has suggested that TGFβ3 might function as a gatekeeper controlling downstream signaling despite high expression of TGFβ1 and TGFβ2 isoforms [53]. Our result showed a connection between a high protein level expression of TGFβ3 and two adverse prognostic markers, HER2 and a high expression of Ki67, but no independent predictive value regarding the time to progression was detected. The result is in agreement with the so far acquired information of the complexity of the TGFβ signalling, the mere amount of immune reactive TGFβ isoform not necessarily illustrating the level of the activity of the pathway.

Foxp3

In our study, Foxp3 was widely expressed in lymphocytes in direct contact with tumor cells (intratumoral) and infiltrating the surrounding stroma (stromal) with no difference in prevalence between primary tumors and recurrent or metastatic lesions, nor between the Groups with different times to progression. Hence, our result showing no predictive value as for the time to progression is in accordance with the previous data showing variable association with good or poor prognosis depending on the tumor type [54]. The mechanisms maintaining peripheral T cell tolerance overlap with those involved in tumor-induced development of its micro environment, and the transcription factor Foxp3 is crucial for the process. It can be induced in the thymus at the presence of interleukin-2 (IL-2), or in the peripheral immune system under the influence of TGFβ and IL-2 [55,56]. In our study the number of intratumoral Foxp3 positive lymphocytes followed the expression of TGFβ1 in the cancer cells, suggesting that in early breast cancer the TGFβ pathway, clearly involved in the development of the tumor micro environment [57], may also be the primary inducer of Foxp3 positive Tregs.

Our results show a significantly increased expression of TGFβ1 in recurrent lesions of breast cancer as compared to the corresponding primary tumor, suggesting that TGFβ pathway is an important factor in the transition of local breast cancer into a disseminated disease, where the option of complete cure is lost. An adjuvant treatment blocking the activation of TGFβ pathway should be considered at the time of the removal of the primary lesion.

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Availability of Data: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval: The Ethics Committee of Helsinki University Central Hospital approved the study protocol.

Authors' contributions

Kristiina Joensuu: Planning the study, responsible for the collection and handling of the samples, evaluating immunohistochemical stainings, making the statistical work, writing the article.

Marja Heiskala: Doing the literature search, planning the study, evaluating the samples, writing the article, preparing the graphics.

Marjut Leidenius: Critical review of the article.

Päivi Heikkilä: Planning the study, analysing the stainings.

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