TGF-ß1 overexpression in breast cancer: Correlation with clinicopathological data

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Abstract. TGF-ß1 belongs to a family of pluripotent growth factors (TGFßs) and has been implicated in the development and progression of human breast cancer. There are conflicting data though, suggesting that TGF-ß has the pontency both to promote and inhibit the progression of mammary neoplasia. We examined the expression of TGF-ß1 mRNA in 24 breast carcinomas using the technique of the reverse transcription polymerase chain reaction (RT-PCR) to obtain quantitative results. Overexpression of TGF-ß1 gene was found in 75% of the cases. We also correlated the overexpression of the TGF-ß1 gene with clinicopathological parameters including histological grade, tumour cellularity, oestrogen receptor status (ER), progesterone receptor status (PR) and lymph node involvement. The results led us to the conclusion that the increasing ratio of overexpression related to the stage of cancer in an analogous way (P~1). No significant association was identified between the ratio of overexpression and the grade, ER, PR, or lymph node involvement (r_s=0.5, 0.2, 0.1, 0.1 respectively; P<0.0001) in all categories.

Introduction

The TGF-ß (transforming growth factors ß) belongs to a family of polypeptides which play a key role in the regulation of a diverse series of processes, e.g. embryogenesis, angiogenesis, inflammation as well as effects on proliferation, extracellular matrix formation and cell migration (1-3).

TGF-ß is a 25 kDa protein, composed of two identical subunits which have been found to be synthesised by a wide variety of normal and neoplastic cells (4). TGF-ß mRNA has been detected in many solid tumours, and there is evidence of altered expression of TGF-ßs in cancer (5). It has been suggested that the growth of these tumours may be influenced by autocrine secretion of TGF-ß and dysregulation of TGF-ß secretion may occur in certain cancers (6).

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The role of TGF-ß in breast cancer is rather controversial as results vary depending on whether cell lines or tissues have been assessed. For example, it was found that there was no difference between mRNA levels in breast cancer cell lines and actively dividing normal cells (7). On the other hand, there is evidence of higher steady-state levels of mRNA in normal cells compared with cancer cell lines (5,8). Analysis of large series of carcinomas and non-neoplastic breast tissue reported levels of TGF-ß mRNA to be higher in the carcinomas (9). We chose to study the expression of TGF-ß1, one of the three isoforms of the TGF-ß indentified in mammals (10), in breast cancer by using RT-PCR. Various studies indicate that TGF-ß1 is an important modulator of angiogenesis (11) with the potential to promote neovascularization in both primary and metastatic breast cancer. TGF-ß1 regulates production of some components of the extracellular matrix as well as increases production of basement membrane-degrading enzymes, which may thereby lead to metastatic phenomena (12-15). Moreover, there are reports on increased growth-inhibitory antiestrogen production of TGF-ß1 in hormonally responsive cell lines (16).

Taking into account that TGF-ß1 along with TGF-ß3 are the most commonly expressed isoforms of TGF-ß in breast cancer (17), our aim was not only to quantify the mRNA levels but also to correlate its expression with clinicopathological data. RT-PCR was used because it is a highly sensitive and rapid technique (18,19). However, some adaptations were required to obtain quantitative results.

Materials and methods

Tissues. Fresh tumour tissue was obtained from mastectomy specimens. Tissues were immediately frozen and stored in liquid nitrogen for later RNA extraction. There were 24 samples obtained from female patients (age range 36-92 years) presented with histologically proven primary breast cancer. In all cases histological confirmation of diagnosis was obtained. With the exception of one case (sample No. 1) all other tissues were malignant.

Tumour stage ranged from T1 to T3 and grade from I-III. Lymph node involvement was determined by histological examination of tissue samples. The oestrogen (ER) and the progesterone receptor (PR) status involvement was obtained from pathology reports (Table I).

Table I. Clinicopathological parameters of 24 patients with primary breast cancer.

Sample No.	Ratio[a]	Stage	TNM	Grade	Hormone receptors ER/PR
1	0.46	Benign			+/-
2	7.2	III	T3N5/35M0	III	+/+
3	0.9	II	T1N0/27M0	I-II	+/+
4	3.2	II	T1.2N0/12M0	II	-/-
5	0.68	*In situ*	T2.5N3/22M0		Non inform.
6	4.0	II	T3.5N0/20M0	II	+/+
7	3.9	II	T1.8N0/23M0	I	Non inform.
8	5.2	III	T2.3N2/16M0	II	+/+
9	7.05	III	T3.5N3/16M0	II	-/+
10	5.6	III	T2.5N8/18M0	II	+/+
11	5.4	III	T2.1N1/11M0	II	+/-
12	3.02	II	T1N0/19M0	II	+/+
13	6.8	III	T2.5N1/21M0	II	+/+
14	7.0	III	T6.7N12/30M0	II	-/+
15	3.7	II	T2.2N0/18M0	II	-/+
16	0.8	I	T0.9N0/13M0	I	+/+
17	3.8	II	T2N0/19M0	II	+/+
18	1.1	I	T1N0/17M0	II	Non inform.
19	2.4	II	T2N0/20M0	II	+/+
20	3.5	II	T1.2N2/19M0	II	-/+
21	1.1	II	T1.3N1/12M0	II	+/-
22	3.5	II	T1.5N1/12M0	II	+/-
23	7.5	III	T3N0/27M0	III	-/-
24	4.5	III	T4N7/30M0	II	+/+

[a]Ratio of the expression of each tumour specimen versus the average expression levels of normal breast tissues. Non inform., non informative.

RNA extraction. Total RNA was extracted using TriZol Reagent (Gibco BRL) following the manufacturer's instructions. RNA content and purity were assessed by spectrophotometry at 260 nm and 280 nm. RNA aliquots (500 ng/µl) were stored at -80°C until cDNA synthesis.

Reverse transcription polymerase chain reaction (RT-PCR). First strand cDNA synthesis. 1 µg of RNA was mixed with 1 mM of each dNTP, 5 mM MgCl, 2.5 µM of oligod$(T)_{16}$, 2.5 U of M-MLV reverse transcriptase in a reverse buffer containing 500 mM KCl, 100 mM Tris-HCl at pH 8.3, in a final volume of 20 µl. The mixture was incubated at 42°C for 30 min, 95°C for 7 min and then at 0°C for 5 min (see below). It was stored at -80°C until used. A negative control without RNA was included.

Polymerase chain reaction. Quantification of TGF-ß1 gene expression was performed after its co-amplification and normalization with an internal control sequence ß2 microglobulin. We chose the ß2 microglobulin instead of ß-actin as a control gene for quantification, based on a recent report that human ß-actin retropseudogenes cause false-positive results in RT-PCR (20). The nucleotide sequence for the TGF-ß1 upstream primer 5'-TAAAAGTGGAGCAGCACGTG-3' and the down-stream primer 5'-GAACCCGTTGATGTCCACTT-3' yielding a 233 bp product. The nucleotide sequence for ß2 microglobulin upstream primer 5'-AAAGATGAGTATGCCT GCCG-3' and the downstream primer 5'-ACTCAATCCAAAT GCGGC-3' yielding a 120 bp product (21).

PCR was performed with an automatic DNA thermal cycler (Perkin Elmer Cetus). Briefly, the reaction mixture was composed of 1/5 of cDNA obtained from 1 µg of RNA, 0.5 µM of each upstream and downstream primers, 50 µM of each dNTP, 1 U of Taq polymerase, 10X PCR buffer containing 500 mM KCl, 100 mM Tris-HCl, 15 HCl at pH 8.3 in a final volume of 50 µl overlayed with mineral oil. After an extensive initial denaturation step (95°C for 8 min) the step cycle program was set as follows: denaturation at 95°C for 50 sec, annealing at 56°C for 40 sec and extension at 72°C for 20 sec for a total of 32 cycles.

Preliminary experiments had revealed the conditions in which the amplification reaction remained in the exponential phase and thus the results could be used for quantification of the TGF-ß1 gene. The optimal conditions consisted of 32 cycles.

The PCR products were visualized on a 2% agarose gel by ethidium bromide staining of the gel. They were also analysed on a 6% polyacrylamide gel which was then stained with the silver staining technique to confirm that there were no non-specific products. Next agarose gels were photographed on a UV transilluminator to analyse the intensity of the bands. The photographs were scanned on a flatbed scanner (Microteck) and computer analysis of the densitometric values were performed.

Statistical analysis. Expression of TGF-ß1 gene was related to clinical and histological parameters and associations were examined using the χ^2 test. Correlations were made by Spearman's rank correlation (r_s).

Results and Discussion

The effects of TGF-ßs on tumor growth are controversial, as they have been reported to inhibit proliferation of epithelial cancer cells (22), and promotion of tumor growth by their angiogenic and immunosuppresive properties (23,24).

The aim of our study was to determine the clinical significance of TGF-ß expression in human breast cancer. Previous studies have based their results on a few well-defined breast cancer cell lines (25,26) or on xenograft tumors (27). Our study was based on 24 tumour specimens from patients with breast cancer. Samples were analysed for the RNA levels

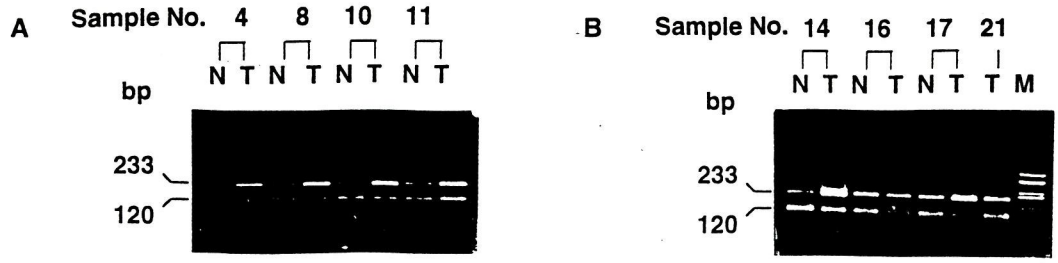

Figure 1. Representantive examples of RT-PCR applied to TGF-ß1 in 8 tumour specimens of breast cancer and 4 normal tissues. In samples No. 4, 8, 10, 11 the same normal tissue was used (A) where in samples No. 14, 16, 17, pairs of normal/malignant tissues from the same patient were used (B).

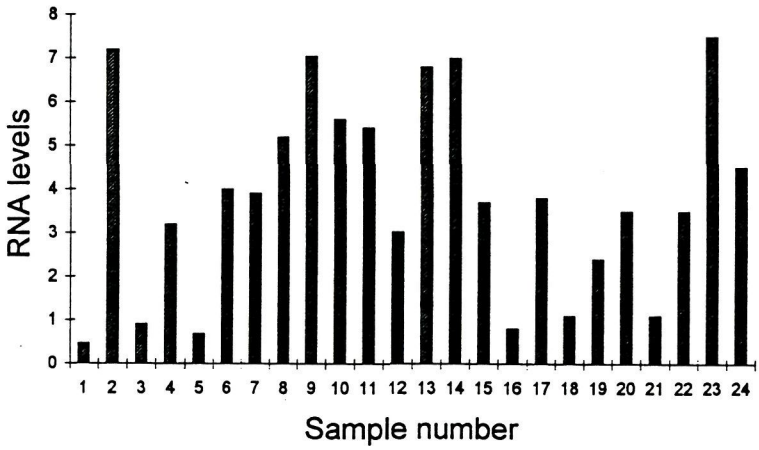

Figure 2. Relative expression of the TGF-ß1 gene. The RNA levels for TGF-ß1 are expressed as the ratios between the intensity of the bands in the tumour tissues, versus the average intensity of the bands in the normal tissues.

of expression of the TGF-ß1 gene, and were interpreted as the ratio of the expression in each tumour specimen versus the average expression level of four normal breast tissues. RNA levels higher than 1.5 fold versus the levels of the control normal tissues were considered an overexpression. Representative results are shown in Fig. 1A and B. TGF-ß1 exhibited overexpression in 18 out of 24 samples (75%), (Fig. 2). Of the 24 cases only 6 were not overexpressed (sample No. 1, 3, 5, 16, 18, 21) (Table I). The mRNA levels for TGF-ß1 was expressed as a ratio of the intensity of bands of the tumor tissues versus the average corresponding levels of the normal tissues. The levels of TGF-ß1 ranged from 0.46 to 7.5. A further analysis revealed the relationship between the rate of overexpression and the stage of tumour. It is noteworthy that for stage I the ratios were 0.8-1.1, for stage II: 0.9-4.0, for stage III: 4.5-7.5 indicating that there was an agreement in the increasing ratio and the increasing stage of cancer. These results are in agreement with those of McCallum *et al* (17) who found levels of TGF-ß higher in malignant than in non-neoplastic tissue (28). i.e. TGF-ß1 is one of the most highly expressed isoforms of TGF-ß in breast carcinomas. These findings are compatible with those previously reported by Arrick *et al* (29) where there was a striking association between the presence of aggressive disease and intense staining for TGF-ß1.

Correlation of the rate of overexpression with clinical data showed that there was no relationship between the rate of overexpression and the grade, the ER and the PR status, neither with the lymph node involvement ($P<0.0001$). The present results are compatible with those previously reported by Barrett-Lee *et al* (9) indicating that there was no significant relationship between RNA levels and histological grade. Thus the levels of TGF-ß1 mRNA may not be simply related to cellular proliferation or the mitotic rate.

Our findings are also compatible with those of Gorsch *et al* (30) where it was found that intense staining for TGF-ß1 was positively associated with rate of disease progression and are independent of age, nodal status or ER status. We disagree with their findings to the point that TGF-ß1 had no association with stage of cancer. This is probably due to the different techniques used.

TGF-ß1 may be used by metastatic cells to achieve anchorage-independent growth. However there is a conflict between the findings of McCallum *et al* (17) and Barrett-Lee *et al* (9) whether TGF-ß1 mRNA is associated with the absence of lymph node metastasis. Unfortunately, in our study no data involving patients with metastatic breast cancer are available.

To determine whether TGF-ß is a useful marker for prognosis in breast carcinomas, longer clinical follow-up as well as larger number of patients are required.

Studies on the regulation of TGF-ß1 gene in breast carcinomas are required to determine whether it is the same or different from normal cells where ECM (extracellular matrix) for example can regulate transcription (31). Furthermore, the investigation of the exact role played by TGF-ß1 with other oncogenes implicated in cancers such as the *nm23* gene which was found to be associated with better survival in breast cancer patients (32) could be informative.

References

1. Barnard JA, Lyons RH and Moses HL: The cell biology of transforming growth factor ß. Biochem Biophys Acta 1032: 79-87, 1990.
2. Ignoz RA and Massague J: Transforming growth factor-ß stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 261: 4337-4345, 1986.
3. Kehrl JH, Taylor A, Kim S-J and Fayci AS: Transforming growth factor-ß is a potent negative regulator of human lymphocytes. Ann NY Acad Sci 628: 345-353, 1991.
4. Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB and Goeddel DV: Human transforming growth factor-ß, complementary DNA sequence and expression in normal and transformed cells. Nature 316: 701-705, 1985.
5. Travers MYT, Barrett-Lee PJ, Berger U, Luqmani YA, Gazet JC, Powles TJ and Coombes RC: Growth factor expression in normal, benign, and malignant breast tissue. Br Med J 296: 1621-1624, 1988.
6. Roberts AB, Thompson NL, Heine U, Flanders C and Sporn MB: Transforming growth factor-ß: possible role in carcinogenesis. Br J Cancer 57: 594-600, 1988.
7. Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS and Berger WH: Synthesis of messenger RNAs for transforming growth factors α and ß and the epidermal growth factor receptor by human tumors. Cancer Res 47: 707-712, 1987.
8. Zajchowski D, Band V, Pauzie N, Tager A, Stampfer M and Sager R: Expression of growth factors and oncogenes in normal and tumour-derived human mammary epithelial cells. Cancer Res 48: 7041-7047, 1988.
9. Barrett-Lee P, Travers M, Luqmani Y and Coombes RC: Transcripts of transforming growth factors in human breast cancer: clinical correlates. Br J Cancer 61: 612-617, 1990.
10. Massagué J: The transforming growth ß family, a review. J Rev Cell Biol 6: 597-641, 1990.
11. Pepper M, Berlin D, Montesam C and Vassali J: Transforming growth factor-ß1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties in endothelial cells *in vitro*. J Cell Biol 111: 743-755, 1990.
12. Salo T, Lyons JG, Rahemtulla K, Birkedal-Hansen H and Larjava H: Transforming growth factor-ß1 up-regulates type IV collagenase expression in cultured human keratinocytes. J Biol Chem 266: 11436-11451, 1991.
13. Laiho M and Keski-Oja J: Growth factors in the regulation of pericellular proteolysis: a review. Cancer Res 49: 2529-2553, 1989.
14. Roberts AB, Sporn M, Assosian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH and Fauci AS: Transforming growth factor ß: rabid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. Proc Natl Acad Sci USA 83: 4167-4171, 1986.
15. Overall CM, Wrana JL and Sodek J: Independent regulation of collagenase, 72-kDa progelatinase and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor ß. J Biol Chem 264: 1860-1869, 1989.
16. Knabbe C, Lippman M, Wakefield L, Flanders KC, Kasid A, Derynck R and Dickson R: Evidence that transforming growth factor ß is hormonally regulated negative growth factor in human breast cancer cells. Cell 48: 417-428, 1987.
17. MacCallum J, Bartlett JMS, Thompson AM, Keen JM, Dixon JM and Miller WR: Expression of transforming growth factor ß mRNA isoforms in human breast cancer. Br J Cancer 69: 1006-1009, 1994.
18. Rappolee DA, Mark D, Bamda MJ and Werb Z: Wound macrophages express TGF-α and other growth factors *in vivo:* analysis of mRNA phenotyping. Science 241: 708-712, 1991.
19. Wanng AM, Doyle MV and Mark DF: Quantification of mRNA by the polymerase chain reaction. Proc Natl Acad Sci USA 86: 9717-9721, 1989.
20. Dirnhofer ST, Berger CHR, Untegasser G, Geley ST and Berger P: Human ß-actin retropseudogenes interfere with RT-PCR. TIG 11: 380-381, 1995.
21. Martyré MC, Romquin N, Le Bousse-Kerdilles MC, Chevillard S and Dupriez B: Transforming growth factor-ß and megakaryocytes in the pathogenesis of idiopathic myelofibrosis. Br J Haematol 88: 9-16, 1994.
22. Lippman ME, Dickson RB, Gelmann EP, Rosen N, *et al:* Growth regulation of human breast carcinoma occurs through regulated growth factor secretion. J Cell Biochem 35: 1-16, 1987.
23. Nitsu Y, Uruschizaki Y, Koshida Y, Terui K, Mahara K, Kohgo Y and Urushizaki I: Expression of TGF-ß gene in adult T cell leukemia. Blood 71: 263-266, 1988.
24. Newcomb SR, Kadin ME and Ansari AA: Production of transforming growth factor-ß activity by Ki-1 positive lymphoma cells and analysis of its role in the regulation of Ki-1 positive lymphoma growth. Am Pathol 131: 569-577, 1988.
25. Bronzert DA, Pantazis P, Antoniades HN, Kasid A, Davidson N, Dickson RB and Lippman ME: Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. Proc Natl Acad Sci USA 84: 5763-5767, 1987.
26. Peres R, Betzholtz B, Westermark B and Heldin CH: Frequent expression of growth factors for mesenchymal cells in human mammary carcinoma cell lines. Cancer Res 47: 3425-3429, 1987.
27. Thompson AM, Steel CM, Foster ME, Kerr D, Paterson D, Deane D, Hawkins RA, Carter DC and Evans HJ: Gene expression in oestrogen-dependent human breast cancer xenograft tumours. Br J Cancer 62: 78-84, 1990.
28. McCune BK, Mullin BR, Flanders KC, Jaffurs WJ, Mullen LT and Sporn M: Localization of transforming growth factor-ß isotypes in lesions of the human breast. Hum Pathol 23: 13-20, 1992.
29. Arrick B, Karc M and Derynck R: Differential regulation of expression of three transforming growth factor ß species in human breast cancer cell lines by estradiol. Cancer Res 50: 299-303, 1990.
30. Gorsh S, Memoli V, Stukel T, Gold L and Arrick B: Immunohistochemical staining for transforming growth factor ß1 associates with disease progression in human breast cancer. Cancer Res 52: 6949-6952, 1992.
31. Struli CH, Schmidhauser C, Kobrin M, Bissell MJ and Deryck R: Extracellular matrix regulates expression of the TGF-ß1 gene. J Cell Biol 120: 253-260, 1995.
32. Barnes R, Masood S, Barker E, *et al:* Low nm23 protein expression in infiltrating ductal carcinomas correlates with reduced patient survival. Am J Pathol 139: 245-250, 1991.