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1. Introduction

Transforming growth factor- β 1 (TGF- β 1) gene expression and protein level in blood as prognostic and diagnostic tools in cancer patients

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Abstract

Aim: To correlate TGF β 1 level of gene expression and protein level in blood together and to the tumor type, size and grade in an attempt to evaluate their use as quick and reliable prognostic and diagnostic tools in solid tumors. And as an important step for targeting TGF β 1 for cancer therapy.

Methods: Blood samples have been obtained from 20 breast cancer (BC) patients and 20 colorectal cancer (CRC) patients. TGF β 1 protein levels were detected in serum after centrifugation by enzyme-linked immunosorbent assay (ELISA); and TGF β 1 gene expression was quantified in whole blood by real time reverse transcription polymerase chain reaction (qRT-PCR). There were 10 healthy subjects served as control.

Results: Serum TGF- β 1 levels in patients with breast cancer (10.42 +/- 2.19 ng/mL) were significantly higher than in normal controls (1.81 +/- 0.22; n = 20) (P < 0.001) and Serum TGF- β 1 levels in patients with CRC (13.9 +/- 2.9 ng/mL) were significantly higher than in normal controls (1.81 +/- 0.29; n = 20) (P < 0.001). TGF- β 1 mRNA in breast cancer patients was significantly higher than in control; about 3 times more abundant in breast cancer patients and about 4 times in CRC patients (P < 0.001).

Serum TGF- β 1 level and gene expression were significantly correlated with clinical tumor size. Serum gene expression levels were correlated with tumor stage and grade, while showing a significant association with distant metastasis (P = 0.006) in breast cancer patients while serum TGF- β 1 level failed to show significant correlation. Neither serum TGF- β 1 level nor gene expression level show significant association with CRC tumor stage, grade, lymph node involvement or distant metastasis.

Conclusion: Serum TGF- β 1 level and gene expression are significantly correlated to solid tumor type and size. Blood TGF- β 1 quantitative gene expression offers an easy, quick and reproducible tool validating serum TGF- β 1 level reliability. TGF- β 1 gene expression showed more significant correlation to tumor grade and metastasis in breast cancer patients.

Impact: This study introduce quick, reliable and reproducible prognostic and diagnostic tool for cancer patients coupling serum TGF- β 1 level to quantitative gene expression in blood samples. Serum TGF- β 1 level previously reported significance as a diagnostic, prognostic and even therapeutic decisive tool is confirmed showing significant correlation to solid tumor type and size. Blood TGF- β 1 quantitative gene expression is offered as an easy, quick and reproducible tool ensuring serum TGF- β 1 level reliability.

Keywords: Transforming growth factor- β 1 (TGF- β 1); Breast cancer; Colorectal cancer.

The transforming growth factor β (TGF β) signaling pathway is a key player growth factor in human biology, and its misregulation can result in tumor development. TGF β has a pleiotropic effect regulating cell growth and differentiation, apoptosis, cell motility, extracellular matrix production and cellular immune responses. TGF β 1 demonstrates paradoxical action exerting tumor-suppressive effects that cancer cells must elude for malignant evolution. Yet, in advanced stages, TGF β modulates processes such as cell invasion, immune regulation, epithelial-mesenchymal transition (EMT), angiogenesis and microenvironment modification promoting the spread of cancers in the body, and can play a direct role in facilitating metastasis. Moreover, TGF β effect is highly contextual influenced by cell type, developmental and disease stage, and innate genetic variation among individuals [1-5]

In breast and colon cancers, TGF β signaling initially has an antineoplastic effect, inhibiting tumor growth, but eventually exerts a proneoplastic effect, increasing motility and cancer spread [6-9]. Breast cancer and colorectal cancer are interesting representatives for solid tumor investigation for a multiple of reasons. Breast cancer is the most commonly occurring cancer among women and colorectal cancer is the third. Both are the second and third leading cause of death after lung cancer. The American Cancer Society has identified colorectal cancer as a major priority because the application of existing knowledge has such great potential to prevent cancer, diminish suffering, and save lives [10]. That is why we have chosen breast and colon cancers being high-frequency tumors with relatively high prevalence in Egypt and Middle East along with growing numbers of survivors, being the most well studied representatives of solid tumors and of the greatest potential for molecular diagnostics and therapeutics studies [11-13]. This was Chittenden and associates' selection for studying somatically mutated genes in some representatives of solid tumors [14].

In recent years, improvements in the understanding of TGF β pivotal role in pathogenesis of a wide variety of diseases including cancer, TGF β 1 offers a prognostic candidate biomarker of tumor invasiveness and metastasis and an attractive, yet challenging, target for cancer therapy [1, 15-17]. One of these challenges lies in the reality that TGF β plays a significant role in its own regulation and thus TGF β message levels do not usually reflect protein production, or, more importantly, protein activity [18]. In this study, we aimed to find out some relation that validates serum TGF β 1 protein level evaluation linked to the message level and correlates to the cancer type, size and grade.

2. Materials and Methods

2.1 Human Subjects and Samples

Twenty female patients diagnosed with breast cancer (11 postmenopausal and 9 premenopausal cases) and twenty colorectal patients (6 females and 14 males) of different tumor grades, with or without metastases were evaluated for their serum TGF- β 1 level and gene expression. Ten healthy individuals (5 females and 5 males) served as controls.

Two blood samples acquired from each; the first was centrifuged and the serum TGF- β level was detected, the second containing whole blood was used for RNA

extraction and quantification.

2.2 Quantitation of human TGF- β level in serum:

Serum TGF- β 1 levels were measured by solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay) technique using Human TGF- β ELISA kit, according to the manufacturer's instructions (ID labs Biotechnology London, ON, Canada) utilizing a monoclonal antibody (capture antibody) specific for human TGF- β coated on a 96-well plate. Standards and samples were added to the wells, and any TGF- β present binds to the immobilized antibody. The wells were washed and biotinylated polyclonal anti-human TGF- β antibody (detection antibody) was added. After a second wash, avidin-horseradish peroxidase was added, producing an antibody-antigen-antibody sandwich. The wells were again washed and a substrate solution was added producing a blue color in direct proportion to the amount of human TGF- β present in the initial sample. The stop buffer was then added to terminate the reaction. This results in a color change from blue to yellow. The wells were then read at 450 nm.

2.3 Quantitation of human TGF- β 1 gene expression using qRT-PCR:

• Total RNA extraction:

Total RNA was extracted from the whole blood using RNA extraction kit provided by Qiagen extraction kit, Germany. RNA purity and quantity were measured by spectrophotometer at 260 nm.

• Primers probes kits:

In this method, quantifast probe assay was used which was provided from Qiagen. This assay is for dual- labeled, probe-based real time-PCR. The assay name is Mm TGF- β 1 QuantiFast Probe Assay (Cat. No: QF00438928). The probe was FAM labeled. The house keeping gene was GAPDH, labeled with MAX (Cat. No: QF00531132).

• qRT-PCR for TGF- β 1 mRNA:

Reverse transcription was carried out in a total volume of 13 μ l. The mix was made of 1 μ g of total RNA and 6.25 μ l master mix-1. The reaction was left to stand for 5 min at room temperature. Then the followings were added: 6.25 μ l master mix-2, 1.25 μ l primer probe, 1.25 μ l GAPDH, 0.25 μ l RT mix (Quantifast) and 3 μ l H₂O. The reaction was carried out using real time PCR at 50°C for 20 min followed by 5 min at 95°C for inactivation of the RT enzyme. The reaction was repeated for 45 cycles of: 95°C for 15 sec and 60 °C for 30 sec.

2.4 Statistical methods:

Data were coded and entered using the statistical package SPSS version 21. Data was summarized using mean \pm standard deviation for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparison between groups was done using analysis of variance (ANOVA) with multiple comparisons post hoc test when comparing more than 2 groups and unpaired T test when comparing 2 groups. For comparing categorical data, Chi square (χ^2) test was performed. Exact test was used instead when the expected frequency is less than 5. P-value less than 0.05 were considered as statistically significant.

3. Results

Serum TGF- β 1 levels in patients with breast cancer (10.42 +/- 2.19 ng/mL) were significantly higher than in normal controls (1.81 +/- 0.22; n = 20) ($P < 0.001$) and Serum TGF- β 1 levels in patients with CRC (13.9 +/- 2.9 ng/mL) were significantly higher than in normal controls (1.81 +/- 0.29; n = 20) ($P < 0.001$). TGF- β 1 mRNA in breast cancer patients was significantly higher than in controls; about 3 times more abundant in breast cancer patients and about 4 times in CRC patients ($P < 0.001$) (Tab.1) (Fig.1)

Serum TGF- β 1 level and gene expression were found significantly correlated with clinical tumor size. (Fig. 2-4) Serum gene expression levels are correlated with tumor stage and grade (Fig. 5), while showing a significant association with distant metastasis ($P = 0.006$) in breast cancer patients (Fig. 6). On the other hand, serum TGF- β 1 level fails to show significant correlation. Neither serum TGF- β 1 level nor gene expression level show significant association with CRC tumor stage, grade, lymph node involvement or distant metastasis.

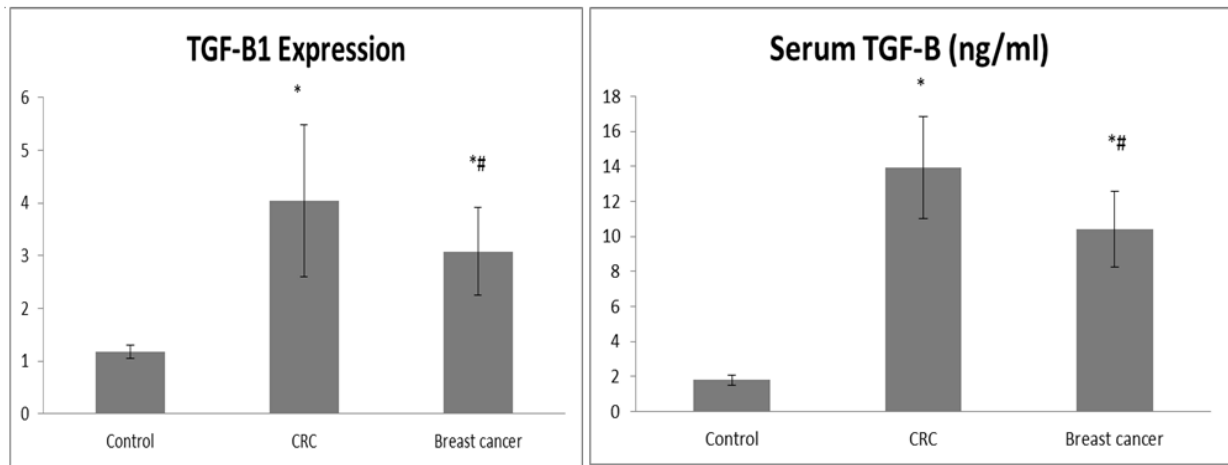


Fig 1: Serum TGF- β 1 levels and gene expression correlated with Cancer type.

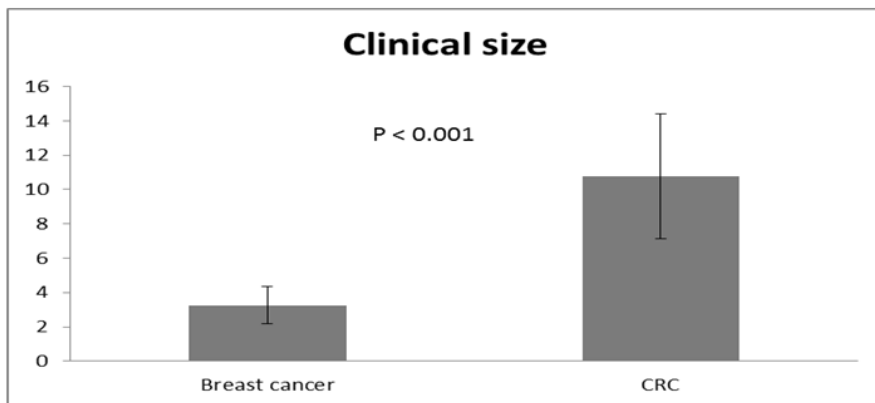


Fig 2: Statistical significant difference between tumor clinical size in breast and colorectal cancer

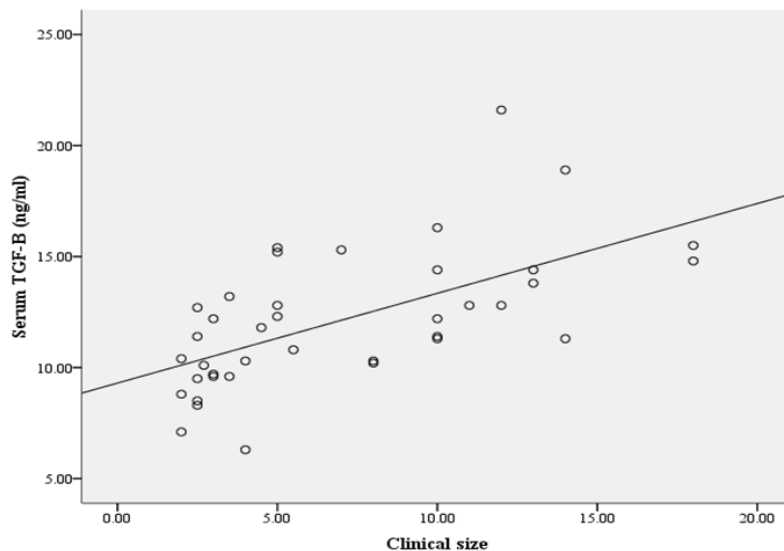


Fig 3: Serum TGF- β 1 levels significantly correlates tumor with clinical size ($P < 0.0001$, n= 39)

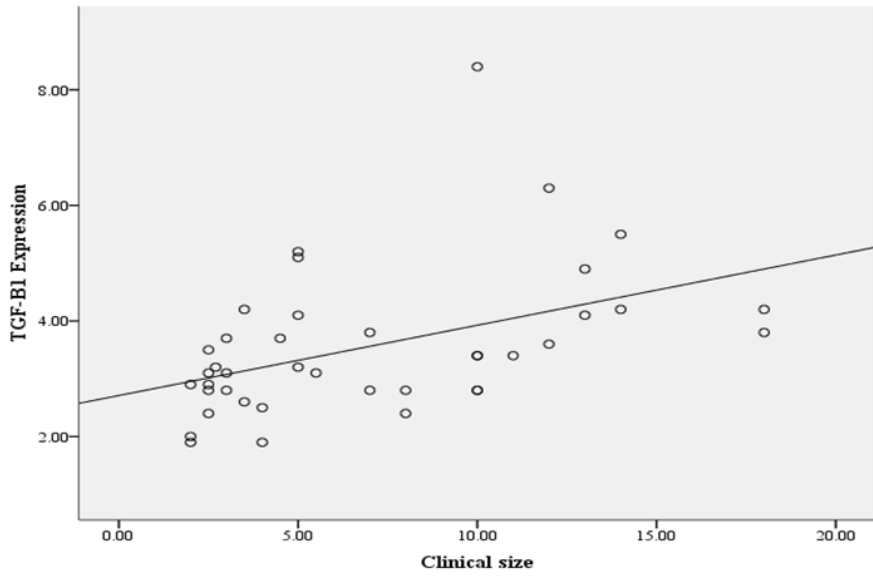


Fig 4: Serum TGF- β 1 levels significantly correlates tumor with clinical size ($P < 0.0001$, $n = 39$).

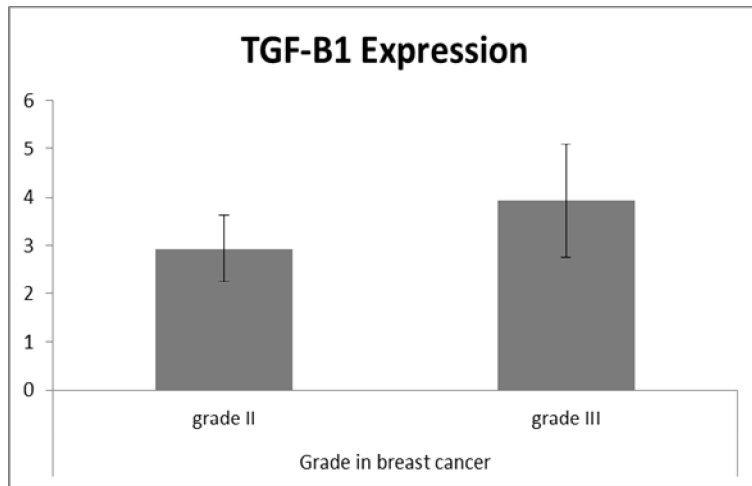


Fig 5: Serum gene expression levels are correlated with tumor stage and grade in breast cancer

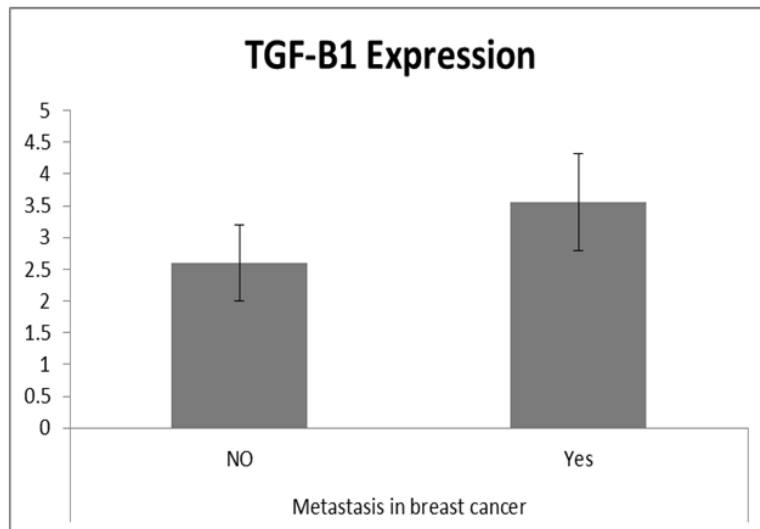


Fig 6: Serum gene expression levels significantly associated with distant metastasis in breast cancer

Table 1 : Serum TGF- β 1 levels and gene expression correlated with Cancer type.
Values are represented as mean \pm SD

	Group			P value
	Control	CRC	Breast cancer	
TGF-B1 Expression	1.18 \pm 0.13	4.04 \pm 1.44 *	3.08 \pm 0.83 *#	< 0.001
Serum TGF-B (ng/ml)	1.81 \pm .29	13.94 \pm 2.90 *	10.42 \pm 2.19 *#	< 0.001

*: statistically significant compared to corresponding value in control group (P<0.05)

#: statistically significant compared to corresponding value in CRC group (P<0.05)

4. Discussion

In Literature, a few studies were found that couple plasma TGF- β 1 mRNA and protein levels as parameters for investigation especially in the field of cancer research. Saigo *et al* used them to study the development of allograft nephropathy in renal transplant recipients [19], Korantzis *et al* used them to investigate the antiangiogenic effect of docetaxel administration in patients with metastatic breast cancer [20]. On the other hand, we found more studies coupling serum TGF- β 1 protein level to tissue gene expression; Tsushima *et al* investigated such parameters to be correlated to cancer degree of progression in Colorectal cancer patients [21]-[24]. Also some studies coupled only one of these two factors to other parameters; Chen *et al* studied only TGF- β 1 mRNA expression in peripheral blood mononuclear cells (PBMC) at different stages of alcoholic liver disease (ALD) to investigate its clinical value [25], Jin *et al* studied Smad7 (one of TGF- β 1 signaling pathway components) RNA expression in peripheral blood mononuclear cells (PBMC) in a set of type 1 diabetes (T1D) patients [26]. Besides, thousands of studies can be found that are based on serum TGF- β 1 protein level investigation. In our study, we decided to couple these two parameters aiming to establishing an easy, quick and reproducible diagnostic tool, prognostic biomarker and even a therapeutic strategy decisive tool to be used in evaluating solid tumors.

In our study, serum TGF- β 1 level in patients with breast cancer and CRC showed significant elevation than in normal controls. That is exactly corresponding to other reports that always showed high serum TGF- β 1 levels with cancer patients yet with variation according to the type [27]. In our study, there is a statistically significant difference between serum TGF- β 1 levels in patients with breast cancer and those with colorectal cancer which showed a significant correlation between serum TGF- β 1 level and gene expression with the solid tumor type.

However, in literature some studies were found as exceptions of this established finding; where there was no difference in serum TGF- β 1 levels in breast cancer patients and normal controls [28, 29]; this was attributed to either the irrelevance of blood samples that have been collected or selection bias.

For the evaluation of the diagnostic significance of serum TGF- β 1, the area under the receiver operating characteristic (ROC) curve (AUC) was 1, while the optimal cut-off point of 4.25ng/ml was determined to classify breast cancer patients, which yielded sensitivity of 100% and specificity of 100% and the optimal cut-off point of 6.2 ng/ml was determined to classify CRC patients, which yielded sensitivity of 100% and specificity of 100%.

Reported cut-off points show great variation in their values for a multiple of reasons; serum TGF- β 1 levels varied considerably between subjects [30]. Given the dual role of TGF- β 1 in

carcinogenesis, a single measurement of serum TGF- β 1 levels at baseline may not be able to capture the critical period involved in multi- stage carcinogenesis [29]. Different adopted techniques for serum TGF- β 1 measurements and/or measuring total or active serum TGF- β 1 justify this great variation in values that varies from 96 pg/ml [31] studying TGF- β 1 levels in predicting post-radiotherapy fibrosis in breast cancer patients to 35.1 ng/ml in CRC patients [23] and 30.86 and 65.15 ng/ml in breast cancer patients [32]. Narai *et al.* reported a correlation between CRC stage and plasma concentration of both active (21.9 +/- 12.8 pg/ml) or total TGF- β 1(18.0 +/- 13.0 ng/ml) despite the variation in values [33].

In this study, the optimal cut-off value in breast cancer patients is comparable to that reported by Demjo *et al.* (3.28 ng/ml) [34] and Ivanovic *et al.* (3 ng/ml) despite using T β RII receptor-based Quantikine TGF- β 1 ELISA kit [35]. And the optimal cut-off value in CRC patients was comparable to that reported by Tsushima *et al.* [36] with a marginal increase in this study's value (7.5 ng/ml) because it correlates plasma TGF- β 1 levels to liver metastasis.

The relation of serum TGF- β 1 levels to tumor stage, grade and progression has been controversially reported. A lot of studies reported no significance with tumor size, stage, lymph node involvement, histological grade, steroid receptor (SR) content in case of breast cancer, and serosal involvement in case of CRC [37-39]. Other studies reported an independent elevation of serum TGF- β 1 [40]. Most reports correlated high serum TGF- β 1 clinical significance to advanced lymph node status, more advanced TNM stage and poor histopathological grade in both breast cancer [32, 41-44] and CRC [22, 23, 30, 33]. Other studies reported the extreme contrary; that low serum TGF- β 1 level is associated with poor prognosis in metastatic breast cancer patients [45] and local recurrence in rectal mucosa [46].

Serum TGF- β 1 level has been widely studied in relation to overall survival in breast cancer patients [35, 37, 41] and in relation to surgical tumor resection in both breast cancer [28, 47] and CRC patients [24, 48].

As discussed before, plasma TGF- β 1 gene expression has been so scarcely reported in cancer oriented studies however elevated tissue gene expression was reported to be associated with tumor stage and disease progression in CRC patients [22, 23] regardless metastasis [24] and with metastasis in breast cancer patients [21].

5. Conclusion and outlook

This study managed to confirm the previously reported significance of serum TGF- β 1 level as a diagnostic, prognostic and even therapeutic decisive tool showing significant correlation to solid tumor type and size. Moreover, it offers blood TGF- β 1 quantitative gene expression as an easy, quick and reproducible tool ensuring serum TGF- β 1 level reliability.

TGF- β 1 gene expression has even showed more significant correlation to tumor grade and metastasis in breast cancer patients.

More studies are needed on larger scale to ensure the reliability of coupling serum TGF- β 1 level evaluation with RNA quantification as a quick and reliable prognostic and diagnostic tool. It has also to be evaluated in relation to overall survival and to responsiveness to therapy either surgical, chemotherapy, or radiotherapy.

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