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Relationship of levels of transforming growth factor-beta1 (TGF-β1) to the levels of ferritin in blood of transfusion dependent β-thalassemia major patients with growth retardation: A case-control study

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Abstract

Introduction: Transforming growth factor-beta (TGF-β) is a pleiotropic polypeptide member of the TGF-β superfamily of cytokines that has multicellular functions. Thalassemias are the most common genetic disorder worldwide. Severe forms are termed thalassemia major characterized by repeated blood transfusions with the elevation of iron levels and progressive multi-organ failure mainly involving endocrine glands and other major organs. It is common to find stunted growth in advanced cases especially among teenage patients. Ferritin is an iron-binding protein can store iron in a safe formula that reflects iron state; conversely, the free-iron concentrations can control intracellular ferritin levels. Serum ferritin is a valuable monitoring marker for iron-overload in thalassemias. This study is designed to assess the relationship of levels ferritin to the TGF-β1 in the sera of β-thalassemia patients and their association to growth retardation. **Materials and methods:** This is a cross-sectional study included 196 subjects; 147 children identified as β- thalassemia major and 49 healthy controls. The thalassemic patients were on consistent follow-up, had received repeated blood transfusions and on iron-chelation therapy. The forty-nine healthy control group were free of any blood disorders or growth abnormalities. The height, weight, and BMI of the studied groups were compared on both standards from WHO and CDC growth charts to evaluate their growth status. In accordance with their growth, the patients were stratified into three groups: those with mild growth impairment, stunted growth, and normal growth groups. Moreover, all subjects' sera concentrations of both ferritin and TGF-β1 were evaluated. **Results:** The mean age showed no significant differences among the 4 groups. The male: female ratio, as well as BMI, were nearly the same amongst the study groups despite both height and weight were significantly differed between normal thalassemics and controls. There were no definite impression of gender on the distribution of ferritin and TGF-β1 individually among the involved subjects. There was a significant correlation of both mean serum ferritin and TGF-β1 mutually, with the four groups being higher in mild and stunted growth patients (2142 ng/ml & 271.3 pg/dl) and lower in normal thalassemics (1005 ng/ml & 76.9 pg/dl) and control group (60.6 ng/ml and 291.7 pg/dl) consequently. The mean ferritin and TGF-β1 levels were significantly correlated only among stunted thalassemics even with levels of ferritin higher than 1000ng/dl. The analysis of linear-regression revealed significant strength of the relationship between levels of ferritin with TGF-β1 in sera of mild and stunted growth patients, while there was a poor insignificant relationship in normal thalassemics and controls. The correlations of ferritin and TGF-β1 with height ZScore and weight ZScore were significant together in 1st and 2nd groups of thalassemia, on the other hand, it was insignificant among third and fourth groups apart from their correlation with height ZScore. **Conclusions:** 1-thalassemia with mild and stunted growth show higher serum levels of TGF-β1 and ferritin than normal thalassemia and healthy subjects. 2-Higher TGF-β1 levels in thalassemics might be an extraordinary cause of disturbing iron metabolism, expressed by elevated serum ferritin levels. 3- Higher TGF-β1 levels correlate well with growth stunting in thalassemic patients.

Keywords: β-thalassemia, TGF-β1, ferritin, growth retardation

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INTRODUCTION

Transforming growth factor-beta (TGF-β) is a pleiotropic peptide member of the TGF-β super-family of cytokines. It represents a cellular protein that achieves

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several cell functions, involving cell-growth regulation, stem cell maturation, besides apoptosis (Ghadami et al. 2000, Vaughn et al. 2000). It has a broad expression namely spleen & bone marrow (Heymann 2014). Recent reports exposed evidence of its role in the control of hematopoietic stem/progenitor cell quiescence, proliferation, and differentiation (Ribeil et al. 2013**)**. TGF-β1 is an inducer of erythroid differentiation, even stronger than erythropoietin at the cellular level (Zermati 2000).

Thalassemias are the most common genetic disorder worldwide, occurs in about 280 x106 people & resulted in 16,800 deaths in 2015 (Vaughn et al. 2000, Heymann 2014). Males and females have similar rates of disease. Patients have defects in either α or β globin chain causing abnormal inherited hemoglobin production. The severity of the two main types, α- and β-thalassemia depends on how many of the 4 genes for α globin or two genes for beta-globin are missing (1). Severe forms are termed thalassemia major characterized by repeated blood transfusions (RBT) with the elevation of iron levels (iron-overload) and progressive multi-organ failure mainly involving endocrine glands and other major organs. Slow growth may occur in thalassemic children and as we know its multifactorial (Ghadami et al. 2000, Ueda and Takasawa 2018). From this time, it is common to find stunted growth in advanced cases especially among teenage patients (Haliloğlu et al. 2017).

Ferritin is an iron-binding protein can store iron in a safe formula express iron state; conversely, the free-iron concentrations can control intracellular ferritin levels. Serum ferritin is a valuable monitoring marker for iron-overload in thalassemias.

Aims of the Study

This study designed to assess the relationship of levels ferritin to the TGF-β1in the sera of β-thalassemia patients (β-TPs) and their association to the growth retardation (GrR).

SUBJECTS AND METHODS

This work was a cross-sectional, accomplished over the six months at Babylon teaching hospital for maternity and pediatrics. The study included 147 children identified as β- thalassemia major (76 males and 71 females) aged between 3-23 years who were attending Babylon thalassemia center. They were on consistent follow-up, had received RBTs, on iron-chelation therapy and being free of chronic hepatitis. Written ethical permission was attained from both the ethical-committee of the hospital and from the parents (or followers) of all sharing children to their child's involvement in the study. The forty-nine healthy control group (HCG) were selected carefully age and sex-matched subjects, all being free of any blood disorders or growth abnormalities. Auxanological parameters were performed for both groups, which included height measurement using standing-stadiometer, weight measurement using accurate scale and BMI calculation (kg /m2). The height and weight of the studied subjects were compared on both standards from WHO growth charts to evaluate their actual growth status. In accordance to their growth, the β-TPs were stratified into three groups: the first group included β-TPs with mild GrR; the second group was stunted growth β-TPs and those with no GrR or normal β-thalassemia (Nβ-TPs) were considered as the third group. Moreover, all subjects' sera concentrations of both ferritin and TGF-β1 were evaluated. Blood ferritin was measured by immunoturbidometric method (Spectrum®, Egypt), and TGF-β1 was evaluated by ELIZA (Elabscience®, China) after venous blood withdrawing.

All the data collected had been analyzed using SPSS version 17 software. Descriptive statistics pertaining to obtained parameters were correspondingly used. A *p*-value less than or equal to 0.05 was considered as significant.

RESULTS

Demographic and Auxanological Parameters

Based on our study results presented in **Table 1**, the mean age (years) of the β-TPs with mild GrR was 11.5, those with stunted growth was 13.9 and Nβ-TPs mean age was 11.6 while, the mean age of HCG was 20 with no significant differences among the 4 groups. The male/female ratio, as well as BMI, were nearly the same amongst the study groups. Even though both height and weight significantly differed in the middle of the β-TPs and HCG.

Biochemical Parameters

The mean serum ferritin and TGF-β1 were 2142 ng/ml and 271.3 pg/dl for those with mild and stunted growth respectively. While the mean serum ferritin and TGF-β1 for Nβ-TPs were 1005 ng/ml and 76.9 pg/dl compared to 60.6 ng/ml and 291.7 pg/dl of HCG consequently. There were no definite impression of gender on the distribution of ferritin and TGF-β1 individually among the involved subjects (**Table 1**). The data generated by this analysis reported in (**Figs. 1** and **2**) display that there was a substantial correlation of both TGF-β1 and ferritin mutually, with the four groups being higher in stunted β-TPs and lower in Nβ-TPs and HCG respectively.

To evaluate the relationships between ferritin and TGF-β1 in studied groups **Figs. A**-**D** were premeditated. In β-TPs, the analysis of linear-regression appear to suggest that there is weak significant strength of the relationship between levels of ferritin with TGF-β1 in sera of both groups of BTPs, R2 = 0.114 and 0.125 (**Figs. A** & **B** respectively). Whereas the linear-regression coefficient of both Nβ-TPs and HCG revealed poor insignificant strength of relationship R2 = 0.001 & 0.065 respectively (**Figs. C** and **D**).

Table 1. Demographic data of the studied subjects involved in the study

Characteristics		β-TPs with mild GrR (N=50)	Stunted β-TPs (N=54)	Nβ-TPs (N=43)	Controls (N=49)	Min	Max	*P*-value
Mean Age/y		11.5	13.9	11.6	20	3	23	> 0.05
Male-Female		23-27	35-19	18-25	26-23			
BMI (kg/m²)		16.01	17.2	30.6	22.8	7.24	26.3	
Height (cm)		134.4	138.2	135.1	169.1	90	187	0.001
Weight (kg)		29.6	33.8	31.4	65.1	8.6	53.2	
Ferritin (ng/ml)	***M***	1274.5	3085.8	993.5	78.2	32	4370	> 0.05
	F	1238.5	2740.2	1013.2	40.7	16	3750	
TGF-β1 (pg/dl)	***M***	269.9	462.7	66.3	317.8	0.25	815.9	> 0.05
	F	240.1	433.7	84.6	256.6	0.37	933.5	

β-TPs: β-thalassemic patients, Nβ-TPs: normal β-thalassemic patients, GrR: growth retardation

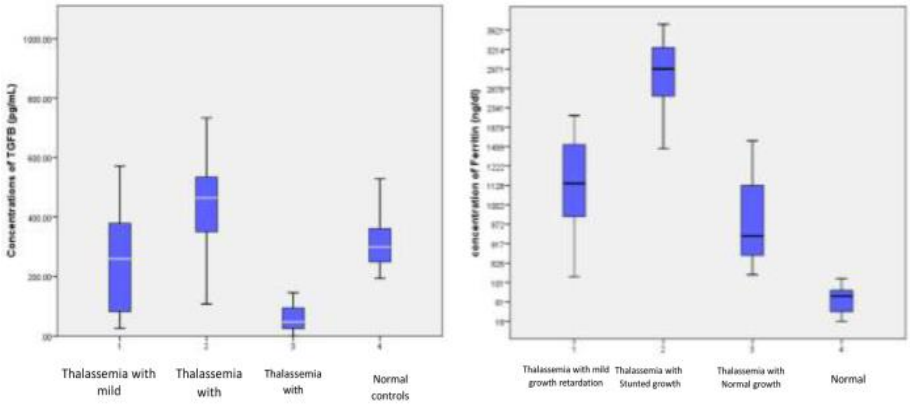

Fig. 1. Correlation of TGF-β1 (pg/ml) and ferritin (ng/dl) in β-thalassemia patients & control

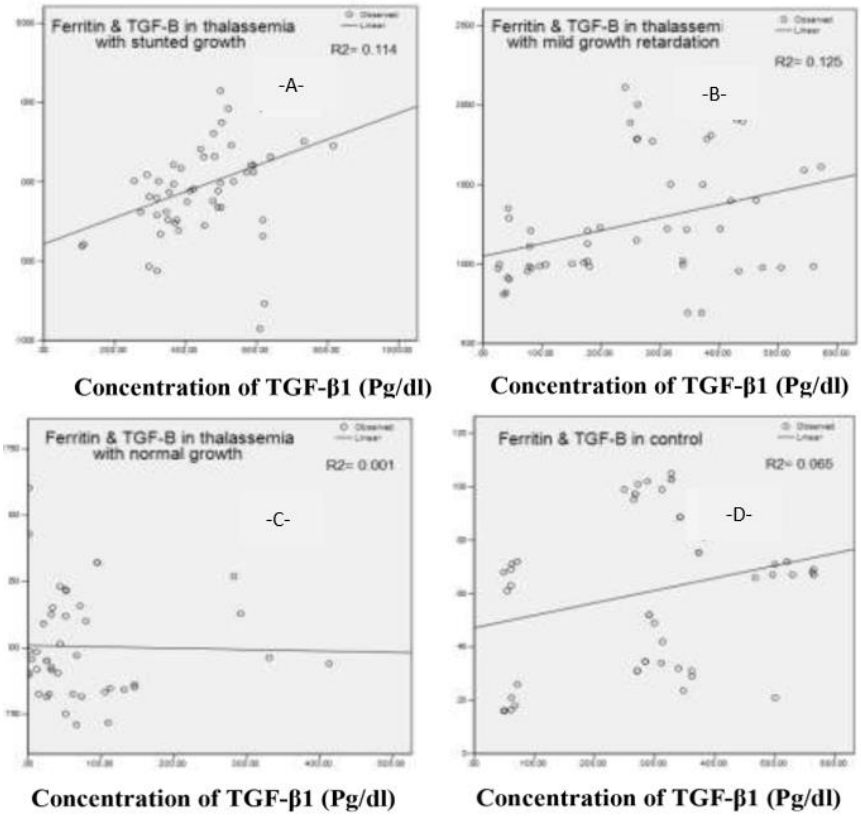

Fig. 2. Regression fit between ferritin levels (ng/ml) and TGF-β1 levels (pg/dl) in the four groups

A closer look at the data exposed by **Table 2** indicates that mean ±SD of ferritin and TGF-β1 serum levels are significantly correlated among β-TPs. This significant correlation is continued with levels of ferritin higher than 1000ng/dl only. The same can not be said for the relation of ferritin and TGF-β1 among Nβ-TPs and HCG where it was statistically insignificant with both higher or lower than 1000ng/dl.

Table 2. Mean and Standard Deviation of Levels of Ferritin (ng/dl) and TGF-β1 (pg/ml) of the Studied Subjects Involved in the Study

		Mean	Std. Deviation	*P-value*
Thalassemia with mild & stunted growth (N=104)	Ferritin	2142	803	0.001
	TGF-β1	271.3	198	
Thalassemia with normal growth (N=43)	Ferritin	1005	197	> 0.05
	TGF-β1	76.9	78.1	
Normal Control (N=49)	Ferritin	60.6	28.7	> 0.05
	TGF-β1	291.7	157	

Table 3. Correlations of ferritin (ng/dl) and TGF-β1 (pg/ml) with height ZScore, weight ZScore & BMI groups in β-thalassemia patients and controls

		Height ZScore	Weight ZScore	BMI Classes
Thalassemiawith stunted growth (N=104)	Ferritin	< 0.05	< 0.05	< 0.05
	TGF- β1	< 0.05	< 0.05	> 0.05
Normal thalassemic (N=43)	Ferritin	> 0.05	> 0.05	> 0.05
	TGF- β1	< 0.05	> 0.05	> 0.05
Control Subjects (N=49)	Ferritin	< 0.05	> 0.05	> 0.05
	TGF- β1	< 0.05	> 0.05	> 0.05

Biochemical and Auxanological Parameters

The correlations of ferritin and TGF-β1 with height ZScore, weight ZScore & BMI groups in β-thalassemia patients and controls can be put forward through **Table 3**. It showed that all three parameters were correlated significantly with serum levels of ferritin and TGF- β1 together in 1st and 2nd groups of BTPs, on the other hand, this correlation is not continued among Nβ-TPs and HCG where it was statistically insignificant apart from their correlation with height ZScore.

DISCUSSION

Growth failure is a common multifactorial complication of BTPs, owing to chronic hypoxia, chronic anemia, intense chelators, reduced level of zinc, iron overload, multiorgan dysfunction and endocrinopathies of several glands such as the pituitary, thyroid, and others (Louis 2005, Seyed Kamal et al. 2018). In the same spirit, the prevalence of GrR in this research was around 63.7%. This rate is differing from what is described in BTPs from different thalassemia centers (Hassan et al. 2018, De Sanctis and Yassin 2019). Such divergence in prevalence can be attributed to numerous factors such as samples' size, high cost of chelation and RBTs, socioeconomic family classes, comorbidities, and others.

In this cross-sectional study, no significant mean age differences among the studied groups. In addition, growth retardation was seen especially in subjects over 10-12 years. These results are comparable to other studies (Louis 2005, Skordis and Kyriakou 2011), but not to others (Hashemi et al. 2011, Shalitin et al. 2005, Tehrani et al. 2019). Additionally, there was no significant gender effect in the incidence of GrR among BTPs that rather like findings of other reports (Shalitin et al. 2005, Al-Sharifi et al. 2019), though other authors revealed some differences between the two sexes (Hammod et al. 2018, Saxena 2003).

Several revisions recognized the high incidence of growth failure in BTPs in children and adolescents concerning intense iron chelators and/or high plasma ferritin levels (Chiou and Connor 2018, Christine et al. 1995, Shalitin et al. 2005). The same effect was noticed in our work, where ferritin values in BTPs with stunted growth and underweight were significantly higher than in HCG. Such a relationship still persevered in BTPs even after they were classified based upon their growth state. This can be explained by a proven effect of iron-chelators on cell multiplication, collagen and DNA synthesis, and deposition of trace minerals like zinc and copper. These complex pathways affect vertebral bodies and resulting in final shortening of the spinal and truncal height (Christine et al. 1995, Low et al. 1998). Other conceivable reasons could be endocrinopathies due to iron accumulation in many endocrine tissues, chronic hypoxia, nutritional deficiencies, liver fibrosis and possibly be due to variation of puberty onset in some BTPs (García-Mayor et al. 1993, Kyriakou and Skordis 2009, Saxena 2003).

For auxanological measurements, the authors rely on the new WHO curves, as they are more sensitive to ascertain weight variation in people at risk, which has crucial effects for preventive and therapeutic purposes (Oliveira et al. 2013).

Iron overload can directly increase serum ferritin (an iron-binding protein) levels. For that reason, the high mean ferritin levels (2114 ng/dl) in stunted BTPs compared to lower ferritin levels in Nβ-TPs and HCG in this study reflect ample support for the claim that the management of BTPs was sub-optimal. This may be double than the desired ferritin values 1000ng/dl (Moiz et al. 2018). Iranian and Turkish analysis reported similar mean serum ferritin levels of 2888 and 1963 ng/dl respectively (Najafipour et al. 2008, Işık Balcı et al. 2016). In the same vein, the mean TGF-β1 level in stunted BTPs was higher compared to levels in Nβ-TPs and HCG also.

The multi-functionality of TGF-β superfamily members has been correlated to more than a few pathological terms and cell process, involving the development of fibrosis and malignancies. The most diversity of precise responses to TGF-β are typically reliant on the TGF-β1 isoform, which is largely studied and more commonly determined member of the superfamily (Tirado-Rodriguez et al. 2014).

In the present study, the issue under scrutiny is the auxanological parameters and its correlation to ferritin and TGF-β1. The data generated by this analysis reported a substantial correlation of ferritin and TGF-β1 mutually with all three parameters among stunted β-TPs, which is not the case in Nβ-TPs and HCG. Debatable results were published last year by other Iraqi researchers from Kerbala city involved both alpha and

beta thalassemia patients that revealed a non-significant negative correlation between TGF-β1 with ferritin while the Turkish researchers three years ago exposed a significantly higher TGF-β1 serum in the patients compared to the healthy controls (Al-Dedah et al. 2018, Baharlou et al. 2016). Not unlike, no significant correlation between TGF- β1 and ferritin was detected in an Iranian study conducted at 2014 to evaluate the impact of silymarin therapy (Balouchi et al. 2014) which might be in part due to immunomodulatory effect of silymarin in addition to its as an iron-chelator. The foregoing unsubstantiated correlation can be put across to some extent keeping in mind several facts.

- TGF-β1 is primarily produced by phagocytic cells or infected somatic cells, its role is important in the differentiation and activation of T-regulatory cells (Treg)48. TGF-β1 is excreted also by antigen-stimulated T-cells56, CD4+ and CD25+ regulatory T-cells (Treg). TGF-β1 inhibits the proliferation of T cells as well as cytokine production via Foxp3-dependent and independent mechanisms. Pro-inflammatory cytokines (e.g. IL-23) and immunosuppressive cytokines (namely TGF-β1) affect the functional status of lymphocytes 26. (Gharagozloo et al. 2009).
- Both Smad 2 and 3 regulate genes transcription induced by TGF-β, those genes play crucial roles in Foxp3-induction and suppression of cytokines, meanwhile; the differentiation of Th17 is enhanced through Smad-independent pathways. This is compatible with Balouchi et al.'s work who reported higher TGF-β1 and IL-17 in thalassemic patients, indicating that Tcells display a overstimulated phenotype, with a paradoxical suppressed activity (Baharlou et al. 2016, Balouchi et al. 2014).
- Additionally, increased TGF-β1 in thalassemia group either of our work or other works (Gharagozloo et al. 2009, Yoshimura and Muto 2011, Chen et al. 2012) reflect that TGF-β1 mainly secreted by Treg, that facilitates immune suppression to limit pathogenesis accompanying chronic inflammatory reaction. Hence, increased TGF-β1 synthesis may result in part to disturbances in iron uptake that can be explained by Th17 overstimulation. In fact, deposition of iron in the reticuloendothelial system may promote Th17 responses in patients with thalassemia and causing higher cytokines levels. Conversely, RBTs cause a state of continuous alloantigen stimulation, in thalassemia even with repressed immunity induced by iron-overload (36). As a whole, T lymphocytes seem to be activated in RBTs patients. Contrary wise, RBT and chronic immune activation may induce Treg cells that may play a role in suppression of T-cell effector functions (Baharlou et al. 2016).
- TGF-β1 is a well known potent inhibitor of proliferation of hematopoietic stem cell (HSC) in vitro, with an unknown role in vivo. (Singbrant et al. 2009).
- Recent researches revealed that signaling of TGF-β1 acts as a double-edged sword, activation or suppression of which can lead to disparity in signaling pathways and cell populations necessary for maintaining normal hemopoiesis (Naka and Hirao 2017, Anya et al 2018).
- TGF-β1 enhances the proliferation of fibroblasts and synthesis of the extracellular matrix, through collagen formation by marrow fibroblasts. Additionally, TGF-β1 mediates atypical stromal signaling that may inhibit or arrest terminal differentiation of erythroid series. Therefore, TGF-β1 is a classical target for both the inhibition of pathological fibrosis and stimulation of erythropoiesis (Naymagon and Mascarenhas 2017).
- TGF-β1/bone morphogenic protein (BMP) signal pathway is intricate in most of the cell activities and is vital during lifetime. TGF-β-BMPs have commonly known contributions in osteogenesis and can regulate several body processes. Signal-transduction by TGF-β-BMPs is carried out by conventional "Smad independent" pathways. Subsequently, in cooperation Smad and p38 MAPK mechanisms congregate at "Runx2-gene" to regulate differentiating stem cells of mesenchyma. The harmonized action of the gene and activated Smads is essential for the skeletal synthesis during embryogenesis. TGF-β1 stimulation by autocrine and paracrine effects is vital for the maintenance and growth of ancestors of osteoblastic cells. Both bones and cartilages express high concentrations of TGF-β1 and target cells for its activity. At early embryological phases, fetal osteoblasts are highly sensitive to TGF-β1 mitogenesis than comparable cells from neonates (Shalitin et al. 2005). Moreover, TGF-β1 signalling pathway enhances progenitor expansion, initial differentiation, and commitment to the osteoblastic series (Chen et al. 2012). This, in turn, might predispose to a state of premature closure of epiphyseal cartilage of axial bones resulting in truncal shortening.

CONCLUSIONS

- B-TPs with stunted growth show higher serum levels of TGF-β1 and ferritin than Nβ-TPs and HCG subjects.
- Higher TGF-β1 levels in thalassemic patients might be an extraordinary cause of disturbing iron metabolism, expressed by elevated serum ferritin levels.
- Higher TGF-β1 levels correlate well with growth stunting in thalassemic patients.

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