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Research Article

Prognostic Relevance of Tumor Necrosis Factor Alpha (TNF-α) and Beta 2 Microglobulin (B2M) in Chronic Myeloid Leukemia (CML)

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Abstract: Tumor necrosis factor alpha (TNF-α) is a major regulatory cytokine which stimulates proliferation of dividing cells while inducing apoptosis in mature progeny. Beta 2 microglobulin (B2M/ β2M), a known prognostic factor in multiple myeloma, reflects tumor burden and turnover. Few in-vitro and clinical studies have demonstrated conflicting observations regarding levels and prognostic significance of TNF-α in CML. Studies have shown prognostic relevance and association with disease stage for B2M in CML. The present study was conducted in the department of Biochemistry in collaboration with Department of Medicine (Clinical Haematology Unit); Pt. B. D. Sharma PGIMS, Rohtak. Levels of TNF-α & Beta 2 Macroglobulin (B2M)were studied in thirty newly diagnosed cases of MBCR-ABL positive chronic myeloid leukemia, confirmed with real time PCR (Polymerase Chain Reaction).Both TNF-α& B2M were estimated in cases before and after chemotherapy (imatinib mesylate). 30 age and sex matched healthy controls were also taken. Initial TNF-α and B2M levels were significantly raised in CML cases in comparison to controls (TNF-α94.48±25.60pg/mL vs not-detectable or 8 pg/ml and B2M 2.47±1.32mg/mL vs 0.99±0.67mg/mL, $p<0.001$ respectively). Both baseline TNF-α& B2M levels at diagnosis were significantly higher in patients not achieving remission after 6 months of imatinib therapy than levels in patients achieving remission ($p=0.019$ & 0.02). B2M levels were significantly correlated with TLC ($r=0.543$; $p=0.004$). Levels of TNF & B2M decreased significantly after therapy in remission group. Thus, TNF-α& B2M levels may help predict non-responding CML patients, studies in larger number of patients are required to validate the observations.

Keywords: Tumor necrosis factor alpha (TNF-α), Beta 2 microglobulin (B2M), imatinib, chemotherapy

INTRODUCTION

Chronic myeloid leukemia is a myeloproliferative disorder characterized by infiltration of the blood, bone marrow and other tissues by neoplastic cells of the hematopoietic system resulting in anaemia, extreme blood granulocytosis, granulocytic immaturity, basophilia, thrombocytosis and splenomegaly.[1] A characteristic chromosomal translocation called the Philadelphia chromosome has been linked with development of chronic myeloid leukemia (CML). Hematopoietic cells contain the fusion gene bcr-abl which encodes a constitutively active tyrosine kinase responsible for the initiation and maintenance of the chronic phase of CML. As hematopoiesis is finely tuned by homeostatic feedback mechanisms involving cytokines and growth factors that modulate the production of red & white cells, and platelets in the marrow cytokines like Tumor necrosis factor (TNF-α) has been implicated in CML pathogenesis[1,2].

Tumor necrosis factor (TNF-α formerly known as tumor necrosis factor alpha) is a major effector and regulatory cytokine that stimulate the acute phase reaction and systemic inflammation[3,4]. It has a pleiotropic role in the pathogenesis of several immune-regulated diseases and hematologic malignancies. It stimulates the proliferation of dividing cells, causing hypercellularity or inducing apoptosis in their maturing progeny, which results in pancytopenia[5]. It is produced by monocytes and T cells but is present in all types of leukemia[6]. It has been demonstrated in, in-vitro studies that TNF-α reduces the viability of leukemic blasts and reduces proliferative response to GM-CSF[7,8,9]. However, other studies showed that TNF-α causes proliferation of leukemic blasts and up-

regulates GM-CSF and IL-3 receptors on them[10,11,12]. While some studies showed no significant changes in TNF-α levels in CML, elevated levels and with prognostic significance has been documented in CML patients in others[13,14,15,16]. TNF-α has also been reported to inhibit the growth of both normal and leukemic hemopoietic progenitor cells in CML[17]. Imatinib mesylate which inhibits TNF-α production in vitro, have potent anti-inflammatory effects[18]. Hence, the in-vitro and clinical studies have demonstrated conflicting observations regarding levels and prognostic significance of TNF-α in CML.

Beta 2 Microglobulin (also known as B2M/ β2M), is a low molecular weight (11.8kDa) protein on the cell surfaces of all nucleated cells and shed into the blood, particularly by B-lymphocytes and some tumor cells. Its levels are increased in multiple myeloma where it is a known prognostic factor.[3] Increased levels of B2M in CML patients have been documented in multiple studies[19,20,21,22]. Peruccio et al. suggested that B2M associated with HLA molecules may represent markers of leukemic blast activation and/or maturation state. B2M may also reflect membrane turnover which is associated with tumor mass and growth rate. Thus, TNF-α and B2M are the upcoming markers for CML[23].

AIMS AND OBJECTIVES:

This study was planned to evaluate the status of TNF-α and B2M in CML patients before and after chemotherapy.

MATERIAL AND METHODS:

The present study was conducted in the Department of Biochemistry in collaboration with Department of Medicine (Clinical Haematology unit); Pt. B.D. Sharma Post Graduate Institute of Medical Sciences, Rohtak. Thirty cases of MBCR-ABL positive chronic myeloid leukemia were taken up for study. The diagnosis was made by real time PCR using commercial kit from Ipsogen. The history, clinical examination, total and differential leukocyte count, bone marrow examination and cytogenetic studies were also reported. Thirty age and sex matched controls were also taken up. CML patients were treated by imatinib therapy[2,24]. Routine biochemistry, serum TNF-α and B2M were performed in newly diagnosed patients before treatment and in controls. The tests were repeated in CML patients after 6 months of chemotherapy or first complete remission (whichever is earlier).

Fasting early morning venous blood sample was taken in a plain evacuated blood collection tube under all aseptic precautions. Samples were processed within one hour of collection. Serum was separated by centrifugation at 3000 rpm X 10 minutes after clotting. Separated serum was stored at -20^0C (maximum 3 months) for serum TNF-α and seum B2M estimation.

Serum TNF-α levels was estimated by a commercial Enzyme Linked Immunosorbent Assay kit for human TNF-α [25]. The lower limit of detection level is 8 pg/ml and the reference range is being even lower.

Serum B2M levels was estimated by a commercial Enzyme Linked Immunosorbent Assay kit for human B2M (DRG β2-MG ELISA) [26]. Its reference range is <2μg/ml; the later is being the lower limit of detection.

STATISTICAL ANALYSIS

IBM SPSS ver. 20 was used for various statistical analyses. Comparison of data between groups was done using 't' test / Mann Whitney Test for quantitative data and Chi-square test for qualitative data. Comparison between multiple groups was done using one-way anova / Kruskal wallis test. Paired samples were compared by paired 't' test / Wilcoxon sign test.

RESULTS:

Both cases and controls had similar age and sex distribution. Median age at diagnosis was 40 years. Median duration of history of presenting illness was 6 weeks. 4 out of 30 patients of CML were asymptomatic and diagnosed incidentally on routine lab examination (Table 1). Median haemoglobin levels were 8.0 g/dL. Median total leucocyte count (TLC) was 80,000 /cu.mm. Patients had 6% blasts in peripheral blood at diagnosis. 26 of 30 patients (86.6%) achieved remission at 6 months of therapy (Table 1). Baseline TNF-α levels were significantly raised in CML cases in comparision to controls (94.48±25.60pg/mL vs not-detectable or 8 pg/ml). Initial B2M levels were also significantly raised than controls (2.47±1.32mg/mL vs 0.99±0.67mg/mL, $p<0.001$) (Table 1). Both baseline TNF-α& B2M levels at diagnosis were significantly higher in patients not achieving remission after 6 months of imatinib therapy than levels in patients achieving remission ($p=0.019$ & 0.02) (Table 2)(Figure 1). B2M levels were significantly correlated with TLC ($r=0.543$; $p=0.004$). (Figure 2) Levels of TNF-α& B2M decreased significantly after therapy only in remission group (Table 3).

Table 1: Demographic and baseline details of CML cases in comparision to controls

	Controls (n=30)	**CML cases before treatment (n=30)**	**p value**
Median duration of illness (months)	-	6	-
Asymptomatic	-	4 (13.3%)	-
Males	16(53.33%)	18(60%)	NS
Females	14(46.66%)	12 (40%)	NS
Age (years) [Mean ± SD]*	37.2±11.17	38.10±12.06	NS
Hemoglobin (gm%) [Median (IQR)]**	13.45(2)	8.43(2)	<0.001
TLC [Median (IQR)]**	7300(2867)	80,000(45000)	<0.001
Blast cells (mean%)		6%	<0.001
Splenomegaly	-	73.30%	-
Hepatomegaly	-	66.70%	-
TNF-α (in pg/ml) [Mean ± SD]*	<8(not detectable)	94.48±25.60	HS
Beta2microglobulin (in µg/ml) [Mean ± SD]*	0.99±0.67	2.47±1.32	< 0.001

NS= Not significant; HS= Highly Significant.

* Mean has been reported with standard deviation (SD)

** Median has been reported with interquartile range (IQR)

Table 2: Baseline TNF-α and B2M in cases and controls

	Control (n=30)	**CML cases(n=30)**		
		Remission (n=26)	**No remission (n=4)**	**p value**
TNF-α (pg/ml)	Not detectable (8pg/ml)	87.08±16.14	142.53±26.70	0.019
B2M (µg/ml)	0.99±0.67	2.10±0.92	4.91±0.57	0.02

The TNF-α and B2M were significantly raised (p<0.005) in both remission and no remission groups when compared with controls.

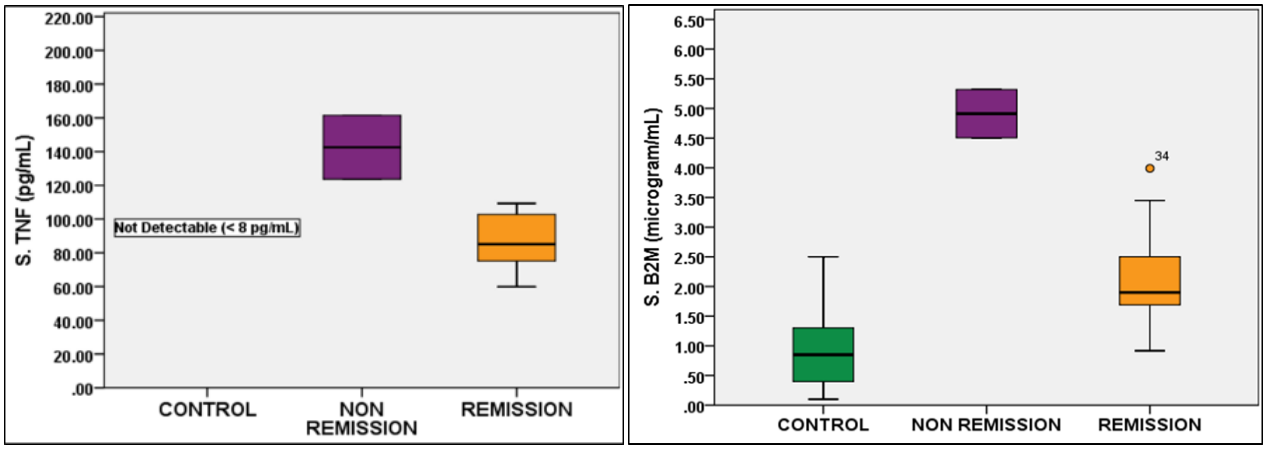

Fig-1: TNF-α and B2M in controls and cases (in both remission and non remission groups)

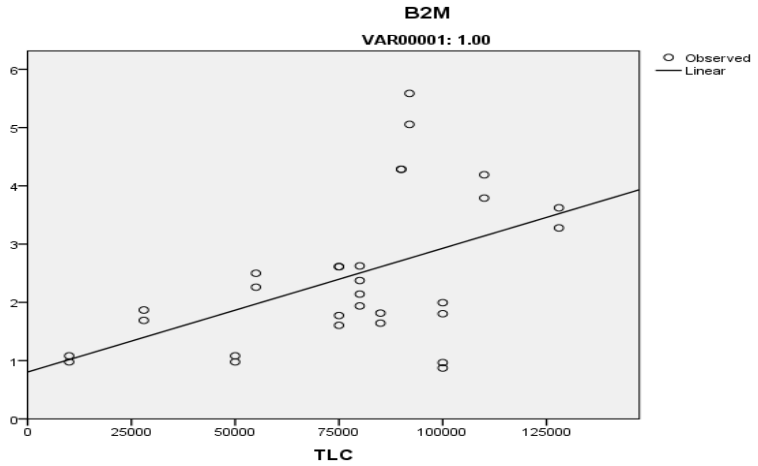

Fig-2: Correlation of B2M with TLC in CML cases

Table 3: comparison of TNF-α and B2M levels in remission and non remission groups in CML

Remission ((n=26)			
	Before chemotherapy [Mean ± SD]	**After chemotherapy [Mean ± SD]**	**p value**
TNF-α (pg/ml)	87.08±16.14	14.97±3.82	0.001
B2M (µg/ml)	2.10±0.92	1.22±0.41	0.009
No Remission (n=4)			
	Before chemotherapy [Mean ± SD]	**After chemotherapy [Mean ± SD]**	**p value**
TNF-α (pg/ml)	142.53±26.70	100.19±4.86	0.180
B2M (µg/ml)	4.91±0.57	3.79±0.12	0.180

DISCUSSION

In the present study there were 9 (60%) male and 6 (40%) female patients. Median age at diagnosis was 40 years with male: female preponderance of 1.5:1 among cases. In general, the male predominance has been estimated to be 1.3-1.4:1[2,24.]. Modak et al. also described highest incidence in the age group of 36 -45 years[27].

Median duration of history of presenting illness was 6 weeks in CML patients. Malaise and fever were most common presenting complaints in 86.7% and 80% cases respectively which are being followed by spleenomegaly, hepatomegaly and bleeding in 73.3%, 66.7% and 13.3% cases respectively. 4 (13.33%) out of 30 patients of CML were asymptomatic and diagnosed incidentally on routine lab examination. The clinical onset of the chronic phase is generally insidious. Accordingly, some patients are diagnosed, while still asymptomatic, during health-screening tests[24].

Median haemoglobin levels were 8.0 g/dL and median total leucocyte count (TLC) 80,000 /cu.mm at presentation. Patients had 6% blasts in peripheral blood at diagnosis. The total leukocyte count was elevated in all the cases at the time of diagnosis and is nearly always greater than 25,000/cu.mm.

In present study, we find that baseline TNF-α levels (i.e. at the time of diagnosis) were significantly higher in cases (94.48±25.60 pg/mL) than in controls (<8 pg/ml) and decreased after 6 months of chemotherapy (14.97 ± 3.82 pg/mL) only on remission. The levels were higher in non-responder at the time of diagnosis (142.53 ± 26.70 pg/mL) as well as after 6 months of chemotherapy (100.19 ± 4.86 pg/mL).

Table 4 : Status of serum TNF-α in CML in various studies

AUTHOR	**FINDINGS**	
Singer et al[13]	No change	No change after therapy
Kiersnowska-Rogowska et al[15]	Increased	
Anand et al[14]	No change	No change after therapy
Hermann et al[16]	Higher levels in non-responders (to IFN alpha-2b therapy than responders and controls	
Osama et al[28]	Increased	Higher levels with advanced disease
Present study	Increased	Higher levels in patients not achieving remission, levels decreased with remission

Osama et al. found serum TNF-α levels increase in patients with both acute and chronic leukemias especially in those with advanced disease[28]. Kiersnowska-Rogowska et al. also detected increased TNF-α concentration in serum of patients with chronic myelogenic leukemia[15]. Herrmann et al. analysed circulating TNF-α levels in 14 patients with CML-CP undergoing IFN alfa-2b therapy. Levels (mean ± SEM) of circulating TNF-α were higher (p less than 0.001) in the group of patients who did not respond to IFN alfa-2b treatment (157 ± 15 U/mL) than in the responders (10.3 ± 4 U/mL) or healthy control subjects (9.1 ± 3 U/mL). However, there was no correlation between TNF-α serum levels and other patient characteristics at study enrolment including age, sex, duration of disease, performance status, splenomegaly, WBC count, platelet count, hemoglobin value, prior therapy, and prognostic category.[16]Our study also found higher levels in non-responders both at the time of diagnosis and after 6 months of chemotherapy. Levels decreased only with remission.

Singer et al. described that on comparing 25 CML patients with ten healthy control subjects, TNF-α levels were not significantly different from controls and reduction in the levels of TNF-α after therapy were non-significant[13]. Anand et al. found that TNF-α levels in CML-CP and CML-BC were not significantly different from those in normal controls. Serum TNF-α levels did not show any variation between CML in remission, CML in relapse and CML in chronic phase. There was no association of TNF-α levels with any clinical feature, spleen size, hemoglobin, TLC, blasts counts, basophil counts, albumin, and bone marrow blasts[14]. The present study also did not find any correlation with other parameters. Thus, high TNF-α levels can help predict non-responders and have prognostic relevance.

Schulz et al. investigated the expression of TNF-α in human leukemic cells by RT-PCR and by a cytoplasmic protein assay. Most cases of CML (n = 5), both in chronic phase and during blast crisis, expressed the mRNA for TNF-α. Their data show that most leukemic cells express the mRNA TNF-α[29]. Liu et al. showed TNF-α mRNA was transcribed in T cells from all of the 12 CML patients studied[30].

Duncombe et al. found that TNF-α inhibits the growth of both normal and leukemic hemopoietic progenitor cells. Exogenous TNF-α reduced the viability and DNA synthesis of purified myeloid cells from patients with CML and inhibited myeloid colony formation by patient progenitor cells. However, unlike progenitor cells from normal donors, patient myeloid progenitor cells also constitutively expressed mRNA for TNF-α and secrete functional TNF-α protein in culture. This endogenous TNF-α impeded the growth of CML cells because anti-TNF-α mAb shown to neutralize bioactive human TNF-α increases CML cell DNA synthesis whereas non-neutralizing anti-TNF-α mAb had no effect. TNF-α-mediated autocrine growth inhibition may contribute to the maintenance of the stable, chronic phase of this disease[17].

Wolf et al. described that the Tyrosine kinase inhibitor imatinib mesylate potently inhibits LPS- and ConA-induced TNF-α production by human myeloid cells in vitro (peripheral blood mononuclear cells, CD14-selected monocytes, and monocyte-derived macrophages) suggesting that imatinib has potent anti-inflammatory effects[18]. In the present study TNF-α levels were decreased significantly by imatinib therapy in the patients achieving remission.

In present study, we find that baseline B2M levels (i.e. at the time of diagnosis) were significantly higher in cases (2.47 ± 1.32 µg/mL) than in controls (0.99 ± 0.67 µg/mL) and decreased after 6 months of chemotherapy (1.22 ± 0.41 µg/mL) on remission. However, in non-responder the levels were higher at the time of diagnosis (4.91 ± 0.57 µg/mL) as well as after 6 months of chemotherapy (3.79 ± 0.12 µg/mL). In addition, B2M levels were significantly correlated with total leucocyte count(r=0.543; p=0.004).

Table 5: Status of serum B2M in CML in various studies

AUTHOR	**FINDINGS**	
Zhara et al[20]	Increased	Higher in blast crisis and accelerated phase
Rodriguez et al[21]	Increased	Associated age, spleen size TLC, percentage blast, lesser chances of remission
Bourantas et al[22]	Levels increased with transformation to blast crisis	
Ellegaard et al [31]	Increased	No change with remission or relapse
Norfolx et al[19]	Increased	
Present study	Increased	Higher in non- responders, correlated with TLC, decreased with remission

Zhara et al found that B2M showed significant increase in the blastic phase, accelerated phase and chronic phase ($p < 0.001$) respectively in study included 50 CML patients[20] . Rodriguez et al. investigated the prognostic significance of serum B2M levels among 201 patients with CML treated with IFN alpha-based therapy. Their median B2M was 2.2 µg/mL (range 1.1-20 µg/mL). Serum B2M levels were associated with other variables of prognostic significance, including age, spleen size, WBC count, percentage of peripheral and marrow blasts, and percentage of marrow basophils. Patients with B2M levels >2.9 µg/mL (ie.,

the upper quartile of the distribution) had a significantly lower rate of major cytogenetic response compared to those in the lower three quartiles (20 versus 52%; P < 0.01). They also had a shorter survival, with a 5-year survival rate of 48%, compared with 75% for those in the lower quartiles (P = 0.01)[21].

Bourantas et al investigated a potential role of B2M in the pathogenesis of myeloproliferative disorders and measured B2M, in 55 patients with myeloproliferative disorders at diagnosis and during the course of the disease. In progressive disease and particularly when transformation to acute leukemia occurred, high levels of B2M were found in all patients; the elevation was progressive, which suggests a potential prognostic usefulness in the individual patient[22]. The present study also found elevated levels with higher levels in non-responders and significant decrease after remission at 6 months. Levels were also correlated with TLC. This reflects the association of B2M with leukemic cell turnover and its prognostic relevance.

Ellegaard et al. and Norfolx et al. also found increased levels in CML patients and concluded these raised serum levels are probably derived from increased cell turnover[19,31].

Thus serum TNF-α and B2M levels have prognostic value in AML patients. Elevated levels of B2M indicated high turnover of leukemic cells and low levels after chemotherapy may indicate the completeness of remission in terms of the leukemic cell turnover better than the absolute cell counts in blood. Thus high TNF-α levels in CML patients can help predict non-responders and have prognostic relevance. Further studies in larger number of patients with long term follow up are required to validate these findings.

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