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sVCAM-1, and TGFβ1 in chronic phase, chronic myeloid leukemia patients treated with tyrosine kinase inhibitors

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Abstract

The outcome for chronic phase (CP) chronic myelogenous leukemia (CML) patients has changed dramatically since the introduction of tyrosine kinase inhibitor (TKI) therapy. We examined the characteristics of CML patients during TKI therapy by determining the plasma concentrations of soluble vascular cell adhesion molecule 1 (sVCAM-1), and transforming growth factor (TGFβ1) biomarkers. The plasma levels of sVCAM-1 and TGFβ1 were measured by ELISA at baseline and after 3 months of TKI treatment. The levels of sVCAM-1, and TGFβ1 were significantly elevated in patients with CML ($P< 0.01$). Dasatinib treatment was associated with a significant reduction in the levels of these biomarkers ($P< 0.01$). In conclusion, plasma levels of sVCAM-1 and TGFβ1 could have a role in the pathogenesis of CML and may be used as predictors of hematological and molecular responses to TKIs. A favorable outcome for Dasatinib therapy was observed.

Keywords: CML, TKI, TGFβ1, sVCAM-1, Imatinib, Dasatinib, Nilotinib.

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm characterized by uncontrolled proliferation of immature myeloid cells in the bone marrow and organs. Also, it is characterized by blood leukocytosis by neutrophils in different stages of maturation.[1]

Tyrosine-protein kinase ABL1 (ABL1) expression has increased in many cases of CML in patients with the fusion gene breakpoint cluster region-Abelson murine leukemia virus oncogene homolog 1 (BCR-ABL1). Tyrosine kinase inhibitors, as ABL1 inhibitors, are used for the treatment of CML patients.[2]

First generation treatment Imatinib is advised for patients who are Sokal low risk while Nilotinib for Sokal intermediate or high risk without cardiovascular disease. In failure of

CML treatment, a second or third generation tyrosine kinase inhibitor (TKI) is recommended.[3]

Transforming growth factor β (TGF-β1) cytokine is expressed throughout the hematopoietic system. It helps suppression of T cell proliferation and also supports differentiation of T cells into specialized T cell subsets.[4] TGF-1 drives the conversion of Foxp3-negative T cells into Foxp3-positive Tregs, which is required for Treg activity maintenance.[5]

TGF-β1 is an important cytokine that promotes B cell and T helper cell interaction, promotes and inhibits immune responses through modulation of immune cells functions. Inhibition of immune responses is achieved by TGF-β1. TGF-β1 suppresses the functions of Th1 and Th2, NK cells, and CD4+ effector cells, and promotes generation of Treg cells. While promotion of immune responses; TGF-β1 induces the generation of Th1 cells in combination with IL-6.[6]

The T helper 3 (TH3) cytokine (TGF-β1) produces autoreactive T cells that inhibit both Th1-and Th2-mediated autoimmune diseases.[7] TGF-β1 is a bystander immune suppressor associated with remission of immune thrombocytopenia.[8] While increased CD11a mean fluorescence intensity (MFI) in CD3+T lymphocytes of immune thrombocytopenia (ITP) patients may cause resistance to immunosuppressive therapy.[9]

Platelet-leukocyte conjugate formation in myeloproliferative disorders induces platelet activation and vascular dysfunction-related biomarkers such as soluble vascular cell adhesion molecule-1 (sVCAM-1) are increased, with a higher chance of arterial or venous thrombotic events than the general population.[10]

VCAM-1 is a cytokine-inducible endothelial cell adhesion molecule (CAM) expressed in two alternatively spliced versions, present primarily in a seven-domain form rather than the rarer six-domain form.[11] sVCAM-1 is elevated in persons living with HIV compared to uninfected individuals.[12] In a study done in Indonesia, which included 68 chronically infected HBV patients, it was found that TGF-β1 levels were significantly elevated in patients with HBV infection.[13]

TGF-β1 and interleukin-10 (IL-10) are key regulators of immune homeostasis.[14] IL-10 has a significant tumorigenesis role, and there is an association between high IL-10 level and advanced stage and other prognostic markers in patients with multiple myeloma.[15] TB+/HCV+ coinfected patients showed higher serum levels of IL-10.[16] The aim of the current study was to describe the plasma levels of sVCAM-1, and TGF-β1 in TKI-treated patients with chronic phase CML and to clarify the clinical significance of these biomarkers during treatment. For avoidance of misleading results, all HBV, HCV and/or HIV positive patients were excluded in the current study.

Subjects and Methods

Ethical statement

The study protocol and methodology were reviewed and approved by the Medical Ethics Committee of Faculty of Medicine, Assiut University, Egypt, (approval No. 17100763, dated April 2019). All available lines of treatment for CML were explained to each patient before enrollment in the study with explanations of the advantages and disadvantages of each line. The right to withdraw from the study at any time was also emphasized. An informed written consent in Arabic language (the native language of the study participants) was obtained from each subject involved in the study before enrollment.

The current study is a cross-sectional, prospective case-control study conducted at Assiut University Hospital, Assiut, Egypt, from May 2019 to Jan 2022. The study included 72 newly diagnosed myeloid leukemia patients in the chronic phase, aged 18:58 years (44 males and 28 females), attended the Clinical Hematology unit of Internal Medicine Department, Assiut University Hospital, Assiut, Egypt. Patients were divided into 3 groups according to TKI treatment. Group 1, CML patients who received Imatinib, Group 2, CML patients received Nilotinib, and Group 3, CML patients received Dasatinib. A control group of 36 normal volunteers, age, and sex matched with the patients, was included in the study.

The exclusion criteria included: CML patients who have previously been treated with TKI, HCV and/or HBV positive patients, HIV positive patients, CML patients with non CML-related anemias, patients with end organ failure, and patients with comorbidities to interfere with treatment.

The three groups of CML patients and the control volunteers were compared regarding age, sex, and serum biochemistry data. Moreover, the levels of biomarkers transforming growth factor B1 (TGF-B1), soluble vascular cell adhesion molecule (sVSAM1) were compared between the groups. Baseline data were obtained before TKI treatment.

Methodology

All subjects included in this study were subjected to the following: history taking including name, age, sex, job, address, special habits as smoking. They were asked for complaint and its onset and duration, especially pallor, fatigue, weight loss, malaise, easy satiety, and left upper quadrant, fullness or pain, bleeding tendency, fever, and recurrent infection. They were then subjected to through clinical examination and laboratory investigations.

Laboratory investigations

Complete blood count (CBC) and peripheral blood film using an automated hematology system (Advia 2120i, Siemens Healthineers, Erlangen, Germany), according to the manufacturer's instructions. The blood film was stained by Leishman staining.

Serum iron, total iron binding capacity (TIBC), ferritin, and Coombs test were performed to exclude causes of non-CML related anemia.

Serological tests for hepatitis B and C, and HIV were done using an immunoassay system (Centaur XPT, Siemens Healthcare Diagnostic), according to the manufacturer's instructions.

Liver function and renal function tests were performed using an automated blood chemistry devise (ADVIA1800 chemistry, Siemens Healthineers, Erlangen, Germany), according to the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The BCR-ABL 1 was performed to help diagnose and monitor the treatment of CML. BCR-ABL 1 was performed using ipsogen BCR-ABL 1 Mbcr IS-MMR commercial kit (Qiagen GmbH, QIAGEN strasse 1, 40724 Hilden, Germany). The kit was performed using QuantStudio 5 Dx system Real-time PCR (Applied Bio systems, Thermo Fisher Scientific, Waltham, MA USA). RNA extraction was performed, followed by cDNA synthesis and finally quantitative PCR was performed using SYBR Green I dye (Thermo Fisher Scientific, USA).

Transforming growth factor B1 (TGF-β1)

TGF-β1 was done using a commercially available kit (TGF-β1 ELISA Kit, SinoGeneClon Biotech Co., Ltd, China), according to the manufacturer's instructions. The end product was measured at a wavelength of 450 nm using a fully automated microtiter plate reader (DSX best 2000®, Werfen, Bedford, MA, USA). The concentration of TGF-β1 in the samples was then determined by comparing the O.D. of the samples to the standard curve.

Soluble vascular cell adhesion molecule (sVCAM-1)

sVCAM-1 was done by using a commercial ELISA kit (sVCAM-1 ELISA Kit, SinoGeneClon Biotech Co., Ltd., China), according to the manufacturer's instructions. The end product was measured at a wavelength of 450nm.The concentration of sVCAM-1 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Assessment of the response to TKI was made clinically and hematologically by CBC and quantitative RT-PCR. After 3 months patients were reassessed again by repeating the following: medical history and clinical examination: by recording size of spleen and liver, anemic symptoms, fatigue, weight loss, infections, and side effects of TKI. CBC was performed twice, after 3-months and again after 6-months. Liver and kidney function tests were measured to assess complications.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 25.0 was used for data management and data analysis. Mean ± standard deviation. Numbers with percentages describe qualitative data. The chi-square test was used for comparing independent categorical variables and Fisher's exact test used in the comparison of the groups. The main outcome variables were shown not to follow a normal distribution. The Mann-Whitney test or Krulis-wallis test were performed for numerical variables not displaying normal distributions. Independent t-tests or ANOVA tests were used to compare means of 2 or >2 independent groups when numerical variables were displaying normal distributions. Spearman correlation coefficients were used to correlate between both biomarkers and other parameters. A *P* value of <0.05 was considered significant.

Results

The demographic and laboratory characteristics of CML patients and controls are shown in Table 1 and Table 2. There was no difference between study groups regarding age, gender, baseline platelet count (Plt; ×103/µl) and mean corpuscular volume (MCV) (*P*>0.05). However, there were significance differences concerning baseline hemoglobin (Hb; g/dl) and white blood cells (WBCs; ×103/µl) (*P*<0.001). All cases and controls were HBV, HCV, HIV negative.

Table 1. Demographic data of the study groups.

Items	Group				*P* value
	Case No.=72		Control No.=36		
Age (Mean ±SD)	48.22 ±8.85		48.33 ±10.0		NS
Gender	n	%	n	%	
male	44	61.1%	20	55.5%	NS
female	28	38.9%	16	44.4%	

Values are presented as mean ± SD, number, and percentage. *P* > 0.05 is not significant (NS).

Table 2. Laboratory data of the study groups.

Items	Group				*P* value
	Case No.=72		Control No.=36		
	Mean	SD	Mean	SD	
Hb (g/dl)	9.27	1.43	12.37	1.06	<0.001
WBCs (×10^3/µl)	207.35	107.36	8.35	1.39	<0.001
Plt (×10^3/µl)	282.65	125.08	319.00	70.22	NS
MCV	89.25	4.94	89.06	3.92	NS
	No.	%	No.	%	
HBV negative	72	100%	36	100%	
HCV negative	72	100%	36	100%	
HIV negative	72	100%	36	100%	

Values are presented as mean ± SD, number, and percentage. *P* > 0.05 is not significant (NS).

A significant difference in the Hb (g/dl), WBCs (×103/µl), and Plt (×103/µl) was recorded after three months treatment in the three groups of CML patients treated with TKI: Imatinib, Nilotinib, and Dasatinib, compared with their respective baseline values. All treatments were equally effective in improvement of laboratory tests with no significant difference between the three studied groups (Table 3 and 4).

Table 3. Demographic data of patients.

Variables	Group						*P* value
	Imatinib No.=28		Nilotinib No.=24		Dasatinib No.=20		
Age (years)	48.76 ±10.6		46.23 ±11.2		49.98 ±10.5		NS
Gender	n	%	n	%	n	%	
male	20	71.4%	16	66.7%	14	70.0%	NS
female	8	28.6%	8	33.3%	6	30.0%	

Values are presented as mean ± SD, number, and percentage. *P* > 0.05 is not significant (NS).

Table 4. Laboratory data during TKI treatment of CML patients.

Variables		Group			*P* value between groups
		Imatinib No.=28 Mean ± SD	Nilotinib No.=24 Mean±SD	Dasatinib No.=20 Mean±SD	
Hb (g/dl)	Before treatment	7.67±1.01	9.69±1.85	9.35±1.30	NS
	After treatment	9.94±0.82	10.69±0.88	10.28±1.07	NS
P value within group		<0.05	<0.05	<0.05	
WBCs ($\times10^3$/µl)	Before treatment	263.0±122.8	168.71±94.89	184.17±88.51	NS
	After treatment	8.61±2.87	8.43±1.35	7.98±2.07	NS
P value within group		<0.05	<0.05	<0.05	
Plt ($\times10^3$/µl)	Before treatment	310.86±122.43	284.14±153.51	224.67±90.71	NS
	After treatment	315.57±81.34	313.14±84.12	286.33±77.57	NS
P value within group		<0.05	<0.05	0.228	
MCV	Before treatment	91.14±1.57	89.43±6.55	86.33±4.80	NS
	After treatment	83.71±4.15	88.43±3.91	84.17±5.49	NS
P value within group		NS	NS	>0.05	

Values are presented as mean ± SD, number, and percentage. *P* > 0.05 is not significant (NS).

The clinical data before and after TKI treatment of CML patients showed significant difference regarding fatigue, pallor, weight loss without trying, bone pain, fever, loss of appetite, spleen diameters in each group except bleeding and bone pain in Nilotinib group and Dasatinib groups and bleeding in Imatinib group (*P*<0.001). There was no difference between three groups regarding all clinical data (*P*>0.05, Table 5).

Table 5. Clinical data during TKI treatment of CML patients.

Variables		Group						*P* value between groups
		Imatinib No.=28		Nilotinib No.=24		Dasatinib No.=20		
Spleen diameter(cm)	Before treatment	4.76 ±2.6		6.67 ±1.2		4.98 ±2.5		NS
	After treatment	2.14 ±1.9		3.76 ±1.7		1.17 ±1.33		NS
P value within group		<0.001		0.001		<0.001		
		n	%	n	%	n	%	
fatigue	Before treatment	28	100.0%	24	100.0%	20	100.0%	NS
	After treatment	10	35.7%	12	50.0%	8	40.0%	NS
P value within group		<0.001		<0.001		<0.001		
Pallor	Before treatment	16	57.1%	12	50.0%	8	44.4%	NS
	After treatment	8	28.6%	4	16.6%	2	10.0%	NS
P value within group		<0.05		<0.01		<0.01		
Weightloss withouttrying	Before treatment	18	64.3%	8	33.3%	8	40.0%	NS
	After treatment	8	28.6%	4	16.7%	4	20.0%	NS
P value within group		<0.05		<0.01		<0.01		
Bone pain	Before treatment	16	57.1%	12	50.0%	8	40.0%	NS
	After treatment	6	21.4%	4	16.7%	2	10.0%	NS
P value within group		0.031		NS		NS		
Easy bleeding	Before treatment	16	57.1%	2	8.3%	8	40.0%	NS
	After treatment	4	14.3%	2	8.3%	2	10.0%	NS
P value within group		NS		NS		NS		
Fever	Before treatment	16	57.1%	6	25.0%	10	50.0%	NS
	After treatment	4	14.3%	2	8.3%	4	20.0%	NS
P value within group		0.016		<0.01		0.016		
Loss of appetite	Before treatment	14	50.0%	8	33.3%	12	60.0%	NS
	After treatment	4	14.3%	2	8.3%	4	20.0%	NS
P value within group		0.031		<0.01		<0.01		

Values are presented as mean ± SD, number, and percentage. *P* > 0.05 is not significant (NS).

Table 6 shows plasma levels of sVCAM1 and TGFβ1, compared between patients with CML and normal volunteers at the start of the study. There was a significance difference between the two groups (*P*<0.001). Table 7 shows the correlation between TGF-B1, sVCAM1 and clinical and laboratory parameters before treatments, including Spleen diameter (cm), WBCs (×103/µl) and BCR-ABL. Table 8 shows a significant decrease in the BCR-ABL, TGF-B1 and sVCAM1 levels, recorded after treatment in three groups, compared with their respective baseline values within each group (p<0.002). Dasatinib treatment was the most effective in decreasing levels of TGF-B1 and sVCAM1 than other treatments.

Table 6. Biomarkers level in the study groups.

Items	Group		*P* value
	Case No.=72 Mean±SD	Control No.=36 Mean±SD	
TGF-B1	0.23±0.05	0.11±0.02	<0.001
sVCAM	0.26±0.07	0.13±0.03	<0.001

Values are presented as mean ± SD or number (%). *P* ≤ 0.05 is significant.

Table 7. Correlation between TGF-B1, sVCAM1 and clinical and laboratory parameters before treatments.

		TGF-B1	sVCA1
Spleen diameter (cm)	r value	0.336	0.408
	P value	0.046	0.011
WBCs ($\times 10^3/\mu l$)	r value	0.410	0.365
	P value	0.005	0.014
BCR-ABL	r value	0.612	0.425
	P value	<0.001	0.010
TGF-B1	r value		0.449**
	P value		<0.001
sVCAM	r value	0.449**	
	P value	<0.001	

**Significant positive moderate correlation. *P* ≤ 0.05 is significant.

Table 8. Biomarkers level during TKI treatment of CML patients.

Variables		Group			*P* value between groups
		Imatinib N=28 Mean±SD	Nilotinib N=24 Mean±SD	Dasatinib N=20 Mean±SD	
BCR-ABL	Before treatment	47.07±15.40	46.25±14.26	46.90±18.92	NS
	After treatment	3.11±3.29	3.20±3.56	2.10±2.64	NS
P value within group		<0.001	<0.001	<0.001	
TGF-B1	Before treatment	0.23±0.03	0.22±0.06	0.25±0.05	NS
	After treatment	0.12±0.02	0.12±0.04	0.09±0.03	<0.01
P value within group		<0.01	<0.01	<0.001	
sVCAM	Before treatment	0.27±0.05	0.26±0.09	0.23±0.06	NS
	After treatment	0.21±0.10	0.19±0.07	0.11±0.05	<0.01
P value within group		<0.01	<0.01	<0.001	

Values are presented as mean ± SD or number (%).*P* > 0.05 is not significant (NS).

Discussion

The present study aimed to assess the plasma levels of sVCAM-1, and TGF-β1 in TKI-treated patients with chronic phase CML and to determine the clinical significance of these biomarkers during treatment. The study included 72 newly diagnosed CML patients and 36 normal controls.

In the current study, the mean age of the patients and controls was 48.22 ±8.85 and 48.33 ±10.0 years, respectively, which was similar to a study done in Sudan, included 99 CML patients;

their mean age was 44.77.[17] In Africa the median age at diagnosis is <50 years, reflecting in part the lower median age of the population.[18] In addition, CML is an age-related neoplasm that commonly happens in older people. The median age at diagnosis was estimated to be 60 years in western countries, but the age at diagnosis in Africa and Asia was about ten years younger.[19]

Besides, CML is more common in males than in females with the male/female ratio of 1.2–1.7. However, to the best of our knowledge, no studies have characterized the distribution and burden of CML worldwide.[20] Regarding gender in this study, 44 patients (61.1%) were male individuals. This agreed with a study done by Rajabto et al., 2022, include 60 patients. Of these, 60% were male and 40% female.[21] The study done in Sudan which included 99 CML patients, indicated that 44.4% were males and 55.6% females.[17]

All CML patients at the start of the study showed CML-related anemia, the mean Hb (g/dl) was 9.27 ±1.43, which goes with findings of a study by Moura et al., 2019.[22] Regarding the mean WBCs (×103/µl) in the current study, it was estimated as 207.35 ±107.36. CML is characterized by a marked leukocytosis of the blood due to neutrophils at different stages of maturation.[7]

In the current study, the clinical presentation of CML patient in the three studied groups included fatigue, pallor, weight loss, bone pain, easy bleeding, fever, loss of appetite, and splenomegaly. The most common symptom was fatigue (100%). Also, Zulbaran-Rojas et al., 2018 reported that fatigue was the most common symptom in all patients prior to the start of therapy.[23] Overall patients tolerated therapy well with improvement in their symptoms from baseline

Splenomegaly (cm) was significantly decreased after administration of treatment in the three studied groups throughout the follow-up period (P<0.001). In the Dasatinib group, there was a significant drop in spleen diameter (cm) from 4.98 ±2.5 to 1.17 ±1.33. Also, for the Nilotinib group, there was a significant drop in spleen diameter (cm) but lower than the Dasatinib group.[23]

A study by Racil et al., 2010, included 20 of 245 newly diagnosed chronic phase CML patients, observed that patients had a palpable spleen at the 3rd month of treatment in terms of treatment response at 18 months after starting Imatinib therapy, and eight of the 20 patients (40%) had achieved a treatment response at these time points.[24] At six months after the start of Imatinib therapy, 11 patients still had palpable splenomegaly, but six of them (54%), at 18 months, still had a therapeutic response, suggesting that slower spleen shrinkage in newly diagnosed chronic phase CML patients does not necessarily mean that the therapy will fail in the future.[24]

As regards laboratory data, we reported that Hb (g/dl) was 7.67 ±1.01 before treatment vs 9.94 ±0.82 after treatment in Imatinib group, 9.69 ±1.85 vs. 10.69±0.88 in Nilotinib group and 9.35 ±1.30 vs 10.28 ±1.07 in Dasatinib group. Concerning WBCs (×103/µl), in Imatinib group the estimated mean was 263.00 ±122.80 before treatment vs. 8.61 ±2.87 after treatment, in Nilotinib 168.71 ±94.89 vs 8.43 ±1.35 and in Dasatinib group 184.17 ±88.51 vs 7.98 ±2.07. Concerning Plt (×103/µl) in Imatinib group was 310.86 ±122.43 before treatment vs 315.57 ±81.34 after treatment, in Nilotinib group 284.14 ±153.51 vs 313.14 ±84.12 and in Dasatinib group 224.67 ±90.71 vs 286.33 ±77.57 before and after treatment, respectively. Also, Nomura et al., 2019 reported that there were no significant differences in the baseline laboratory data, including red blood cells, Hb, WBCs and Plt, between the TKI treated groups.[25] WBCs and Plt showed significant decreases after TKI treatment, while Hb showed significant increases after TKI treatment.[25]

The results of the current study, BCR-ABL after three months of TKI treatments, showed marked decrease in Dasatinib group (2.10 ±2.64), in comparison to Imatinib group (3.11 ±3.29) and Nilotinib group (3.20 ±3.56). Most patients in the chronic phase of CML achieved a major molecular response within three to six months of initiating treatment with TKI.[26]

In the current study, the plasma concentrations of sVCAM1 before treatment were the highest in the Imatinib group (0.27 ±0.05), compared with the Nilotinib group (0.26

±0.09) and Dasatinib group (0.23 ±0.06). The sVCAM1 levels after treatment were significantly decreased in the Dasatinib patients (0.11 ±0.05) than those observed in the Nilotinib patients (0.19 ±0.07) and in the Imatinib group (0.21 ±0.10) ($P< 0.01$). The mean plasma concentrations of TGF-B1 before treatment were 0.23 ±0.03 and 0.22 ±0.06 in Imatinib and Nilotinib groups, respectively. The highest value was in the Dasatinib group (0.25 ±0.05). The TGF-B1 level after treatment was markedly decreased in the Dasatinib group (0.09 ±0.030) compared to other groups. These findings agreed with those reported by Nomura et al., 2019, as after 6 months, the administration of each TKI therapy significantly reduced the plasma concentrations of sVCAM-1, and TGF-B1 compared to baseline levels, indicating inflammation reduction.[25] The changes in Dasatinib-treated patients were markedly different from those in the control group.[25] Dasatinib treatment significantly decreased the levels of sVCAM-1 biomarkers. Dasatinib is a second generation TKI, more potent than Imatinib and active against several Imatinib-resistant BCR-ABL1 mutants.[27]

Furthermore, identifying levels of biomarkers plays a critical role in clinical and laboratory data in CML patients. A significant positive correlation detected between TGF-B1, sVCAM1, spleen diameter (cm), and WBCs (×103/µl). TGF-B1 levels were moderately correlated with spleen diameter (cm) ($r = 0.336$; $P = 0.046$) and WBCs (×103/µl) ($r = 0.410$, $P = 0.005$) among cases, and it was statistically significant. There was a positive moderate correlation between sVCAM1 level and spleen diameter (cm) ($r = 0.408$, $P= 0.011$) in addition to a positive moderate correlation with WBCs (×103/µl) ($r = 0.365$, $P = 0.014$).

In conclusion, data of our study indicated that plasma levels of sVCAM-1 and TGFβ1 could have a role in the pathogenesis of CML and may be used as predictors of hematological and molecular responses to TKIs. A favorable outcome for Dasatinib therapy was observed.

Author Contributions

MRA, YAA, MGE and RB conceived and designed the research. ENA and RB recruited patients. RB and MGE carried out the clinical investigations. MRA, ENA, MGE and YAA collected clinical data. MRA, YAA, MGE, ENA and RB contributed in the interpretation of data for the work. MRA prepared the original draft of the manuscript. All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Declaration of Conflicting Interests

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Ethical approval

The study protocol and methodology were reviewed and approved by the Medical Ethics Committee of Faculty of Medicine, Assiut University, Egypt, (approval No. 17100763, dated April 2019).

Informed consent

An informed written consent in Arabic language (the native language of the study participants) was obtained from each subject involved in the study before enrollment.

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