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Association Between Soluble CD40L with Thrombosis Occurrence and JAK2 V617F Mutation in Essential Thrombocythemia

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Abstract: Thrombo-haemorrhagic events are the main cause of mortality in essential thrombocythemia (ET). The aim of this study was to measure soluble CD40 ligand (sCD40L) in the plasma of healthy individuals and in patients with an elevated platelet count and investigate the association of sCD40L with thrombosis in ET patients and their JAK2 V617F mutation. The plasma levels of sCD40L was measured in 75 patients. 35 patients diagnosed as ET, 25 patients diagnosed as reactive thrombocytosis (RT), 15 patients with low platelet count and 15 healthy subjects acted as the control group. 35 ET patients were assessed for JAK2 V617F status by utilizing a JAK2 V617F specific quenching probe. ET patients had the highest levels of sCD40L compared to the patients with RT and controls (225.70 ± 79.34, 160.40 ± 54.54 and 83.54 ± 21.54) respectively and a tight correlation was found between the platelet count and sCD40L. Statistical analysis revealed that the JAK2 V617F mutation was associated with significantly increased levels of WBCs ($p<0.04$) and sCD40L ($p<0.001$) compared to JAK2 V617F negative patients. There was no significant association between JAK2 V617F mutation and thrombosis, but the level of sCD40L was significantly higher in patients with thrombosis than those without thrombosis (236.43 ± 75.93 vs 184.65 ± 62.31) respectively. Based on these findings, the presence of JAK2 mutation may changes the expression of soluble markers of endothelial and platelet activation besides the quantitative and qualitative changes in platelets. Mechanisms leading to thrombosis are more complex and multifactorial.

Keywords: Essential Thrombocythemia, sCD40L, JAK2 V617F Mutation, Thrombosis

1. Introduction

Essential thrombocythaemia (ET) is a myeloproliferative neoplasm characterized by an increase in the peripheral blood platelet count that is associated with bone marrow megakaryocyte hyperplasia, without associated erythrocytosis or leukoerythroblastosis [1].

According to different reports, 50% to 60% of patients with ET have mutation V617F of the Janus Kinase (JAK2) gene [2]. A single G to T somatic point mutation at nucleotide 1,849 in exon 14 of JAK2 results in the substitution of valine with phenylalanine at codon 617 (JAK2 V617F), consequently increasing the tyrosine kinase activity leading to abnormal expansion of myeloid cells [3-5].

It has been determined that the JAK2 V617F mutation occurs at the stem cell level, and is the major molecular mechanism, as well as a potential diagnostic marker for the development of MPNs, including PV and ET [6-8]. Many studies have been performed to evaluate the association of JAK2 V617F with the risk of thrombosis, especially in patients with ET, but the results have been inconclusive [9-12].

CD40L is expressed in activated CD4 T lymphocytes and platelets and is converted to sCD40L via a proteolytic

process [13]. On platelet activation, P-selectin and CD40 ligand are rapidly translocated to the platelet surface and subsequently cleaved to generate soluble forms fully active biologically, termed soluble P-selectin (sPselectin) and soluble CD40 ligand (sCD40L) [14, 15], both of which promote coagulation by inducing tissue factor (TF) expression on the monocytes and the endothelial cells [16]. Soluble CD40L also causes platelet activation and appears to be required for thrombus formation in vivo [15, 17]. However, the presence of CD40L in platelets makes possible the assumption that circulating sCD40L derives from the platelets and that the observed variations of sCD40L concentrations in disease states are related to the platelet count. These observations provided the rationale for our study. We asked whether there was a relationship between platelet number and concentration of sCD40L in plasma in healthy individuals and in patients with an elevated platelet count.

Considering that thrombosis is a major cause of morbidity and mortality in patients with ET [18] and a remarkable proportion (50-40%) of ET patients are reported to be JAK2 V617F negative cases, yet thrombotic complications can also be observed in this group [19, 20]. So, the identification of a biological marker for high risk patients with ET would be of great clinical utility. Therefore, we investigated the association of sCD40L with thrombosis in ET patients and their JAK2 V617F mutation.

2. Study Design

2.1. Patients and Controls

The study was carried out at the Department of Internal Medicine, Division of Hematology and Clinical Lab, Assiut University Hospital, Assiut, Egypt. The study group included 75 patients. 35 patients were diagnosed as having ET (11 male /24 female) with a mean age of 54.62 ± 7.02. The diagnostic criteria for ET were those recommended and updated by the World Health Organization (WHO) in 2008 [21]. 25 patients (8 male / 17 female) with a mean age of 58.4 ± 7.53 were diagnosed as having RT (patients with a platelet count above 450×10^9/L, without a myeloproliferative disorder, and with an underlying disease likely associated with RT). In each case the platelet count normalized if the acute disease state was no longer present after treatment.

Causes of RT were malignancy (10 patients), infection (8), anemia attributed to iron deficiency (7). 15 patients with low platelet levels ($< 100 \times 10^9$/L) (4 male / 11female) with a mean age of 52.76 ± 7.49 included 2 patients with myelodysplasia, 9 patients with idiopathic thrombocytopenic purpura (ITP), and 4 patients with secondary thrombocytopenia resulting from an autoimmune or lymphoproliferative disorder. Controls were 15 healthy blood donors (5 male/10 female) with a mean age of 45.60 ± 13.12 who were sex and age matched with patients. None of the patients with ET or healthy subjects had symptoms of an acute infection or inflammatory diseases.

The study was approved by the local ethics committee and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients enrolled into this study.

2.2. Laboratory Analyses

Whole blood was routinely collected into tubes containing ethylene diaminetetraacetic acid (Becton Dickinson, Mountain View, CA) and was immediately centrifuged at $3000g$ at 4°C. Plasma samples were a liquated and stored at -80°C until assay. Repeated freezing and thawing process was avoided.

2.2.1. Routine Hematological Assays

White blood cell count, hematocrit, hemoglobin and platelet counts were determined by automated methods using a Cell-Dyn 3500 Analyzer (Abbot).

2.2.2. Soluble CD40L Elisa

Quantification of sCD40L in plasma was performed with a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

2.2.3. JAK2 V617F Mutation Analysis

DNA extraction: Genomic DNA was isolated from whole EDTA blood using QIAamp DNA Mini kit (Qiagen, Germany) according to manufacturer's instructions.

Real time quantitative polymerase chain reaction (RT-PCR) assay for JAK2 V617F mutation.: using Fast 7500 Applied Biosystems. The presence of the *JAK2*V617F mutation was investigated as described by Baxter *et al* [6]. Briefly, 80 ng of DNA from the patients were used to amplify the mutated and unmutated exon 14 of *JAK2* in an allele-specific polymerase chain reaction (PCR). PCR products were separated on a 3% agarose gel, stained with ethidium bromide, and viewed under UV light. A 203 base-pair fragment indicates the presence of the 1849G>T mutation. A quantitative real-time PCR-based allelic discrimination assay was used to detect the *JAK2*V617F mutation employing TaqMan real-time technology. Genomic DNA was amplified in a 40-cycle PCR at an annealing temperature of 61°C. All reactions were carried out in a final volume of 25 μL containing 1x PCR Master Mix (Applied Biosystems), 900 nM of both forward and reverse primers and 100 nM of each probe. A ratio between the Ct (JAK2 V617F) and Ct (JAK2 wild-type) was calculated for each sample. *cDNA pooled from ten Donors were used as the calibrator sample.* The sequences of the primers and probes are listed in table 1.

2.3. Statistical Analyses

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, version 21.0 for Windows, Chicago, Ill, USA). Data were expressed as mean $\pm$ standard deviation (SD). A "p"value <0.05 was accepted as significant. Mann-whitney U test was used to compare

continuous variables and Chi-square to compare categorical variables between the groups. Pearson correlation analysis was applied and Pearson coefficient of correlation (r) was used to show the relationship between the variables.

Table 1. *Sequences of the real-time primers and probes for JAK2.*

5'forword primer	5'reverse primer	5'probe
AAGCTTTCTCACAAGCATTTGGTTT	AGAAAGGCATTAGAAAGCCTGTAGTT	MGB JAK2(A) FAM 5'-TCCACAGAA ACATAC JAK2(C) VIC 5'-CTCCACAGA CACATAC

3. Results

Levels of sCD40L were measured by ELISA in the plasma of 75 patients and 15 healthy subjects act as the control group.

All patients with high platelet counts (RT and ET) had significant raised levels of sCD40L with a mean value of (160.40±54.54 and 225.7±79.34, respectively) when compared with controls (83.54±21.54) and patients with low platelet counts (23.40±4.33). Highest levels of sCD40L were seen within the ET population figure 1. A positive correlation between sCD40L and platelet counts was observed (r=0.889, p<0.000) figure 2.
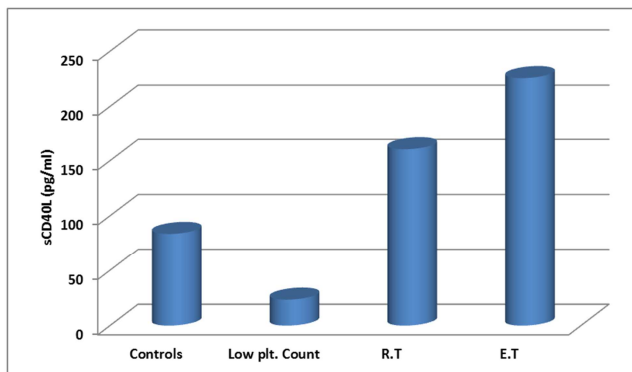

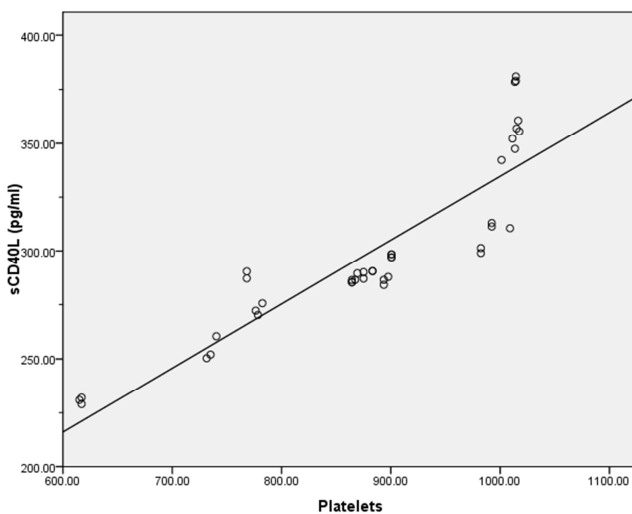***Figure 1.*** *Levels of sCD40L in the patients and healthy controls.*

Figure 2. *Correlations between the platelet count ($*10^9$/L) & the plasma concentrations of sCD40L (r=0.889, P<0.000).*

At the time of study, ET patients received one or more of

the following therapies: anti-platelet agents (aspirin) and myelosuppressive drugs (e.g: hydroxyurea). Patients with ET had significantly higher white blood cells (p<0.02*) and platelet counts (p<0.000***) compared to healthy controls.

The main demographic and clinical data of ET patients and healthy controls were summarized in table 2.

Table 2. *Demographic and clinical data of controls and ET patients.*

Item	ET n =35	Control n =15	p-value
1-Age "years"			
mean±SD	54.62±7.02	45.60±13.12	p=0.483 (n.s)
(min-max)	(48.0-70.0)	(31.0-69.0)	
2-Sex:			
M/F	11(31.42%) /24(68.57%)	5(33.33%) /10(66.67%)	p=0.582 (n.s)
3-Hct"%"	41.32±7.77	40.83±8.56	P=0.667 (n.s)
4-WBCs count ($*10^9$/L)	8.23±2.56	6.43±1.89	p<0.02*
5-Platelets count ($*10^9$/L)	792.54±275.53	242.66±79.32	p<0.000***
6-Splenomegaly	8 (22.85%)	--	--
7-History of DVT	13 (37.14%)	--	--
8-History of bleeding	--	--	--

Data presented as mean ±SD. p<0.05 is significant

Table 3. *Clinical features in 35 patients with ET according to JAK2 mutation.*

Item	Jak2 V617F +ve N=19	Jak2 V617F -ve N=16	p-value
1-Age "years" mean±SD	56.25±12.65	52.43±15.43	P=0.639 n.s
2-Sex:			
M/F	6(31.57%) /13(68.42%)	5(31.25%) /11(68.75%)	P=0.685 n.s
3-Hct"%"	41.76±9.38	40.55±9.16	P=0.582 n.s
4- WBCs count ($*10^9$/L)	9.82±3.04	7.5±2.38	P<0.04*
5- Platelets count ($*10^9$/L)	815.24±201.21	850.42±210.38	P=0.758 n.s
6-Splenomegaly	5 (26.31%)	3 (18.75%)	P=0.582 n.s
7-History of thrombosis	7 (36.84%)	6 (37.50%)	P=0.527 n.s
8-sCD40L(pg/ml)	245.23±115.43	171.22±74.65	P<0.001**

Data presented as mean ±SD. p<0.05 is significant

Detectable amounts of JAK2 V617F DNA in whole blood were found in 19 out of 35 patients with ET (54.28%) figure 3, 4. No mutation was identified in the controls or in any patients with RT, demonstrating the high specificity of the JAK2 V617F mutation in classical MPNs. As summarized in

table 3, the JAK2 V617F positive patients had significantly higher mean WBC and sCD40L than those without the mutation (p<0.04* and p<0.001**, respectively). Thrombotic events were observed in 36.84% of patients with JAK2 V617F compared with 37.50% of patients without JAK2 V617F.
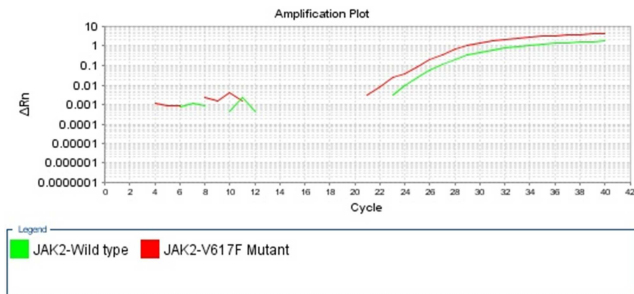

Figure 3. *Patient positive for JAK2 mutation expression.*
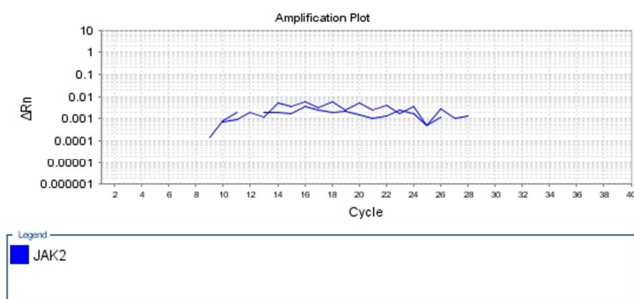

Figure 4. *Patient negative for JAK2 mutation expression.*

We found no statistical difference in age, sex, hematocrit levels, splenomegaly rate, or thrombosis occurrence according to JAK2 mutation status. Patients with a history of thrombosis had significantly higher sCD40L levels than those without thrombosis (236.43 ± 75.93 vs 184.65 ± 62.31, p <0.001*) and controls (83.54±21.54, p<0.001**) figure 5.

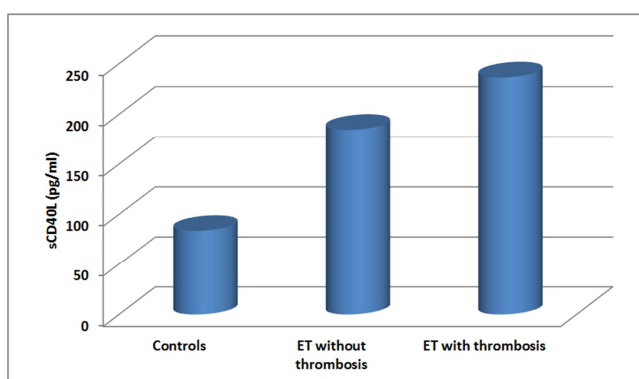**Figure 5.** *Concentration of sCD40L in matched healthy controls and ET patients without or with thrombosis.*

4. Discussion

CD40 ligand (CD40L) is a type II transmembrane protein that is expressed on activated CD4+ T lymphocytes and can be released as a soluble form (sCD40L) by an ill-defined proteolytic process. Although it is known that CD40L is expressed over the surface of activated platelets and that this is followed by a subsequent release of sCD40L from platelets, sCD40L levels may vary according to platelet count and/or the disease state [13, 22].

In our study, sCD40L levels were significantly higher in all patients with high platelet counts compared with controls. Highest levels were seen within the ET population and this higher level showed parallelism with platelet counts, which was consistent with the previous studies [23, 24]. These results revealed that there was a significant correlation between platelet count and sCD40L in patients with ET and that levels were also increased in reactive thrombocytosis, with sCD40L levels being increased when the platelet count is high. The fact that sCD40 levels correlate with platelet count shows that sCD40 is found largely in platelets though expressed in many other cells. A correlation between platelet-associated CD40 and soluble CD40 levels and increased levels of sCD40L, as a marker of platelet regeneration, where there is increase megakaryopoiesis, has also been reported.

Essential Thrombocythemia (ET) is characterized by persistently elevated platelet counts in the context of a normal red cell mass [25]. Several diagnostic algorithms for this disorder have been proposed. However, irrespective of the criteria employed for diagnosis, patients with ET comprise a heterogeneous group. Heterogeneity is manifested both by the variation in clinical course observed, and by the variable presence of cellular and molecular markers [26]. *JAK2 V617F* mutation has been recognized as a possible thrombotic risk factor in ET. Its role is probably due to an increased myeloid proliferation and white blood cells (WBCs) activation [27].

In the present study, screening of JAK2 V617F was performed in 35 patients with ET according to the WHO diagnostic criteria [21, 28], we found a 54.28% incidence of the JAK2 V617F mutation among our ET patients, a finding in agreement with different reports that described point mutation in the JAK2 kinase (JAK2 V617F) in about 30-70% of ET patients [2, 29-31].

In contrast to other investigators [27, 32-34], a non-significant association between the JAK2 V617F mutation and the thrombotic events was detected in current study group. Although the JAK2 V617F mutation was associated with significantly increased levels of WBC counts but its direct effect on the thrombotic events did not prove its suggested predictive potential. However, the present study results indirectly support the concept that the JAK2V617F mutation contributes to intrinsic changes in both megakaryocyte and platelet biology beyond the increase in cell numbers [35–37]. The results of another single center analyses also revealed that the JAK2 V617F (−) cases did not differ significantly from the JAK2 V617F (+) cases in the incidence of thrombosis [38-40]. The number of involved patients might be determinative in view of clear evaluation of the predictive value of the JAK2 V617F mutation in thrombosis [41, 42]. Neither hematocrit levels nor platelet counts showed significant differences in patients with JAK2 V617F mutation than in those without, as reported in

previous studies [12, 34, 43].

The role of sCD40L in the thrombosis of ET was assessed in this study, as well as its relationship with the patients' JAK2 mutation status. Similar to this study, previous studies reported elevated sCD40L levels in ET patients with thrombosis [23, 44]. In addition, sCD40L induces platelet P-selectin expression, aggregation, leukocyte activation, platelet-leukocyte conjugation, and platelet release of reactive oxygen intermediates [15, 16, 45, 46]. Our findings suggest that sCD40L, which are released from the activated platelets, could play a role in the pathogenesis of ET thrombosis.

Our study confirmed the previous finding of increased plasma concentrations of sCD40L in ET patients with the JAK2 mutation [47]. Furthermore, these data analyzed the relationship between JAK2 mutation status and this activation marker according to the proportion of mutant alleles. A JAK2 mutant allele dosage effect could be demonstrated on sP-selectin, sCD40L, TF, and VWF: Ag levels, with the highest plasma levels being found in patients with more than 12% JAK2 V617F mutant alleles, supporting the role for sCD40L in thrombosis of ET as well as the association between JAK2 V617F allele burden and soluble markers of platelet and endothelial activation.

5. Conclusion

In conclusion, the impact of JAK2 V617F mutation in the prediction of subsequent thrombotic events could not be proven in this study. Our results indirectly support the concept that, besides the quantitative and qualitative changes in the platelets, the mechanism leading to thrombosis is more complex with multifactorial contributions.

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