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Heat-shock protein 70 and pentraxin-3 inflammatory biomarkers: Implication for thrombosis in polycythemia vera

Ibrahim Abdullah Mahmood, Farqad Bader Hamdan, Waseem Fadhil Al-Tameemi[1]

Abstract:

BACKGROUND: Chronic inflammation has been suggested to contribute to the pathogenesis of thrombosis in polycythemia vera (PV) as it triggers *in vivo* activation of platelets, leukocytes, and endothelial cells, which are all of major importance during thrombus formation.

OBJECTIVES: The aims of this study were to evaluate the pathophysiology behind increased thrombosis in PV in terms of the effect of *JAK2*^{V617F} gene mutation copies in relation to the intensity of the heat.shock proteins 70 (HSPs70) and long pentraxins.3 (PTX.3).

SUBJECTS AND METHODS: Thirty patients with PV, 23 with secondary polycythemia, and thirty healthy volunteers were studied. Hemoglobin level, packed-cell volume, white and red blood cells count, mean corpuscular volume, and platelet counts were estimated. The enzyme-linked immunosorbent assay was used to estimate the HSP70 and PTX-3 levels, whereas the real-time polymerase chain reaction technique for the assessment of the *JAK2* mutation rate was done for only thirty PV patients.

RESULTS: Significantly higher HSP70 and PTX-3 levels were detected in PV patients. A positive relationship was demonstrated between the *JAK2* mutation rate and each of HSP70 and PTX-3 and between the latter two biomarkers.

CONCLUSION: The elevated HSP70 and PTX-3 concentrations and the clear relationship between them and *JAK2* mutation rate can drive the procoagulant activity in blood cells in patients with PV.

Keywords:

Heat-shock proteins 70, inflammation, *JAK2*, polycythemia vera, PTX-3

Introduction

Polycythemia vera (PV) is classified by the WHO classification system under the major category of myeloproliferative neoplasms (MPNs).[1,2] PV is a stem cell-derived clonal myeloproliferation that is characterized by increased red cell mass on normal hemoglobin (Hb) oxygen saturation, an elevated white cell and platelet count, low erythropoietin (EPO) levels, and exclusive "driver" mutation of *JAK2*,[3] in addition, to significant symptom burden and increased risk for thrombosis, myelofibrosis, and leukemic transformation.[4]

The tendency for thrombosis in PV patients results from a complex interplay of multiple variables.[5,6] Conventionally, erythrocytosis,[7] leukocytosis,[8,9] and aging[8] have been implicated as risk factors of thrombosis. Among the nonconventional risk factors of thrombosis include the *JAK2*^{V617F} allele burden,[10] blood cell activation,[11-13] coagulation system activation,[14] and inflammation.[15]

Studies have provided evidence that the chronic MPNs may be driven by[16] or accompanied by[17,18] chronic inflammation

Departments of Physiology and [1]Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Address for correspondence:
Dr. Farqad Bader Hamdan, Department of Physiology, College of Medicine, Al-Nahrain University, P.O. Box 70044, Baghdad, Iraq.
E-mail: farqadbhamdan@colmed-alnahrain.edu.iq, farqadbhamdan@yahoo.com

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for the clonal change.[19,20] In sequence, chronic inflammation elicits *in vivo* activation of platelets, leukocytes, and endothelial cells (ECs),[21-23] which collectively of great importance during thrombus formation.

Pentraxins-3 (PTX-3), the prototype of the long PTX, is a member of the family of acute-phase proteins that have been significantly linked to a number of foremost thrombotic episodes in patients with PV.[24-26] On the contrary, the highest PTX-3 levels were related to a lower thrombosis rate indicating a protective effect against thrombosis.[15] Actually, its deficiency has been shown to encourage vascular inflammation and atherosclerosis and elevated PTX-3 levels to be linked to a reduced risk of cardiovascular disease and thrombosis.[18]

On the other hand, it has been demonstrated that PTX-3 will increase the tissue factor (TF) expression in mononuclear and ECs. The increased level of TF, the main coordinator of the coagulation cascade, causes the thrombus formation, a feature of atherosclerosis.[27] Heat-shock proteins (HSPs) are regarded as a set of pervasive and highly conserved proteins[28,29] thoroughly synthesized after normal cells exposed to a diversity of physiological and environmental agents such as temperature, stress to hypoxia, inflammation, infections, or anticancer chemotherapy.[30] After exposure to inflammation, their level increased and confers cytoprotection.[31]

This work aimed to evaluate the pathophysiology behind increased thrombosis in PV in terms of:
1. Effect of the *JAK2*^{V617F} gene mutation copies in relation to the intensity of the HSP70 and long PTX-3
2. To compare the above-mentioned parameters in PV with secondary polycythemia as well as healthy controls.

Subjects and Methods

The present study was approved by the Institute Review Board (the local Ethical Committee) of the College of Medicine, Al-Nahrain University (MA10-17/1/2016), Baghdad. All the participants were informed about the study, and their consent was obtained.

Patients group

Fifty-three patients were recruited from those attending the outpatient clinic of the Clinical Hematology in Al-Imamain Al-Khadimiyan Medical City and the National Center for Hematology Diseases and Researches, College of Medicine, Al-Mustansyria University. They comprised 30 patients with PV (13 males and 17 females with an age range between 18 and 78 years) (mean ± standard deviation [SD] = 54.87 ± 13.44 years) and another 23 patients with secondary polycythemia (22 males and one female with an age range between 25 and 65 years) (mean ± SD = 40.13 ± 12.21 years).

All the patients were examined by a specialist/consultant in clinical hematology before being considered eligible for the purpose of the study. Polycythemia had been confirmed clinically and through the laboratory parameters. It is defined as Hb 18.5 g/dL in men, 16.5 g/dL in women, or other evidence of increased red cell volume.

The PV patients' group was subdivided into three subgroups:
1. A long-standing group comprised 13 patients whom they were already diagnosed and under follow-up
2. Those with thrombotic complications comprised seven patients and include any PV patients who had previous or recent thrombotic complications such as stroke, pulmonary embolism, and DVT.
3. The newly diagnosed group comprised ten patients who diagnosed as having PV disease during the period of blood sampling for the current study.

The patients selected to have PV according to the WHO criteria (2008)[32] and those with secondary polycythemia when the PCV ≥52%. All patients with uncontrolled chronic medical diseases such as diabetes mellitus with long-term complications, history of hereditary thrombophilia, or coagulopathy disorders (i.e., hemophilia and Von Willebrand diseases), clinical evidence of acute inflammatory conditions (i.e., acute infections), history suggestive of underlying connective tissue diseases, and any acquired cause for thrombotic complication (i.e., female patients taking contraceptive pills) were excluded from the study.

The control group

Another thirty aged- and sex-matched, nonsmokers healthy volunteers comprised 16 males and 14 females were also studied. Their ages were between 20 and 68 years (mean ± SD = 52.1 ± 11.16 years).

Methods

Blood sampling

For all patients and control groups, complete blood count was done, including Hb level, hematocrit, white blood cell, red blood cells, mean corpuscular volume, and platelets.

Again 5 ml of venous blood samples were aspirated from the antecubital vein using 10 ml disposable syringe, and then, the blood transferred into different tubes:
1. Two milliliters of blood into a tube containing an anticoagulant (EDTA) used for studying the quantitative assessment of *JAK2*^{V617F} mutation in primary polycythemia patients.

The DNA was extracted from whole blood using a ready kit (gSYNC™ DNA Mini Kit Whole Blood Protocol, Geneaid, Korea) according to the manufacturer's instructions. Then, real-time polymerase chain reaction (PCR) was done based on the measurement of accumulated PCR product through a Taqman probe. This TaqMan® probe is a small oligonucleotide labeled with two different fluorescent dyes. In the 5′ end, a reporter dye (6-FAM [6-carboxyfluorescein]) is attached, and in the 3′ end, a quencher dye (CHQ [Black Hole Quencher®]) is attached.

2. Three milliliters of the blood into a centrifuge gel tube (clot activator tube) allowed to clot for 10 min and then centrifuged at 4000 rpm for 5 min, and the serum was collected for the estimation of EPO, HSP70, and PTX-3 using human enzyme-linked immunosorbent assay kits (Elabscience, Wuhan, China).

Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences software (version 16. IBM Inc., Chicago, USA). Continuous variables were expressed by means ± SD, whereas categorical variables were expressed as percentages.

The analysis of variance test was used to compare between different groups where there is a continuous variable, and Pearson's Chi-square was used to compare the percentages of these groups where there are categorical variables. The association between different variables was examined with a binary correlation test. The causal relationships between some variables were investigated with the regression test. $P < 0.05$ was considered statistically significant.

Results

As illustrated in Table 1, EPO level was statistically significantly higher ($P < 0.001$) in patients with secondary polycythemia than patients with PV and controls; meanwhile, no difference was noticed between the latter two groups. HSP70 was statistically significantly different among the three studied groups ($P < 0.001$). PTX-3 was significantly higher ($P < 0.001$) in PV patients as compared to the patients with secondary polycythemia and controls, but no difference in their level was noticed between the latter two groups.

EPO level was statistically significantly lower ($P = 0.004$) in the newly diagnosed PV patients when compared to those with a history of thrombosis or long-standing disease. Both latter groups show no difference in the EPO level. The HSP70 and PTX-3 were not different among the three subgroups ($P = 0.19$ and $P = 0.12$, respectively), as shown in Table 2.

Figures 1-3 showed that *JAK2* was positively correlated with HSP70 and PTX-3 ($P = 0.028$; $P = 0.006$, respectively). Moreover, HSP70 was positively correlated with PTX-3 ($P = 0.035$).

EPO level shows no correlation with HSP70 and PTX-3 in patients in secondary polycythemia and control groups [Table 3].

Discussion

The results of our study demonstrate a significant increment in HSP70 and PTX-3 levels in PV patients compared to the controls and those with secondary polycythemia. HSP70 overexpression in PV was reported by Gallardo *et al*.[33] In secondary polycythemia, HSP70 was remarkably high when compared to that of the control group. This is because EPO "the most important survival factor for erythropoietic development" will positively influence HSP70 activities and/or expression to enhance cell survival.[34,35]

Table 1: Erythropoietin, heat-shock protein-70, and pentraxin-3 levels in patients with polycythemia and controls

Parameters	Polycythemia patients		Controls (n=30)
	Primary (n=30)	Secondary (n=23)	
EPO (pg/ml)	2323.3±656.29	3355±271.05$^{\$}$	2186.3±220.5
HSP70 (ng/ml)	56.46±12.38*	46.9±12.78**	34.74±6.14$^{\#\#}$
PTX-3 (ng/ml)	31.23±6.53$^{\#}$	24.19±5.79	21.64±2.44

$^{\$}P$<0.001 (secondary vs. primary polycythemia and controls), *P<0.001 (primary vs. secondary polycythemia), **P<0.001 (secondary polycythemia vs. controls), $^{\#\#}P$<0.001 (controls vs. primary polycythemia), $^{\#}P$<0.001 (primary vs. secondary polycythemia and controls). EPO=Erythropoietin, HSP70=Heat-shock protein 70, PTX-3=Pentraxin-3

Table 2: Erythropoietin, heat-shock protein-70, and pentraxin-3 levels in polycythemia vera subgroups

Parameters	Polycythemia vera patients		
	Newly diagnosed (n=10)	With thrombosis (n=7)	Long-standing (n=13)
EPO (pg/ml)	1853±485.58$^{\#}$	2838.1±414.14	2407.9±656.99
HSP70 (ng/ml)	57.28±13.09	59.38±11.81	55.03±10.17
PTX-3 (ng/ml)	29.55±5.36	30.64±7.27	27.33±3.21

$^{\#}P$=0.004 (newly diagnosed vs. with thrombosis and long-standing). EPO=Erythropoietin, HSP70=Heat-shock protein 70, PTX-3=Pentraxin-3

Table 3: The correlations between erythropoietin and inflammatory markers in secondary polycythemia and control groups

Parameters	EPO	HSP70	PTX-3
EPO (r, P)			
Polycythemia	1	−0.153, 0.486	0.073, 0.742
Controls	1	0.030, 0.876	−0.048, 0.802
HSP70 (r, P)			
Polycythemia		1	−0.038, 0.863
Controls		1	0.372, 0.423

EPO=Erythropoietin, HSP70=Heat-shock protein 70, PTX-3=Pentraxin-3

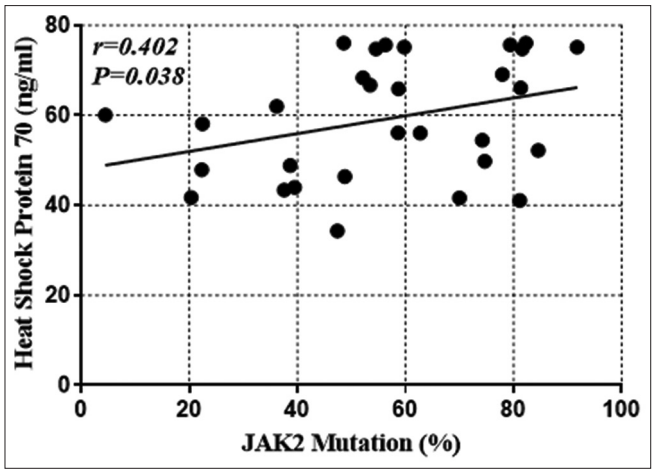

Figure 1: The correlation between *JAK2* mutation rate and heat-shock protein-70 level in polycythemia vera group

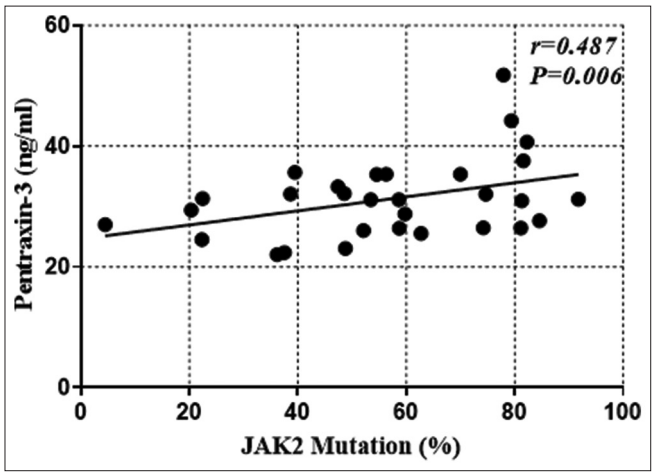

Figure 2: The correlation between *JAK2* mutation rate and pentraxin-3 level in polycythemia vera group

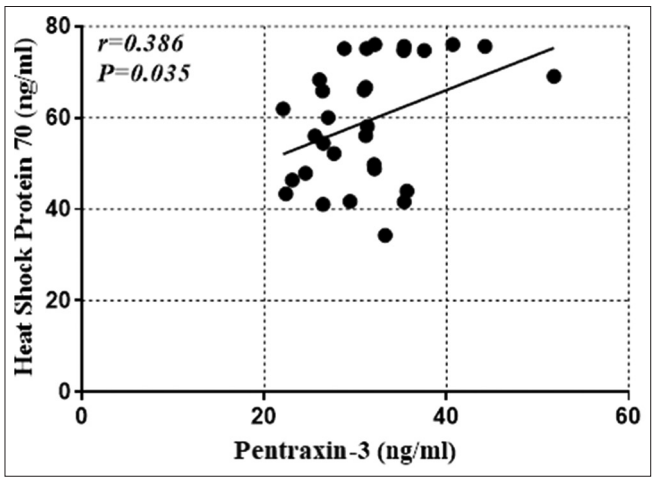

Figure 3: The correlation between heat-shock protein-70 and pentraxin-3 levels in polycythemia vera group

Elevated circulating HSP70 might be reactionary to tissue injury or its release from the damaged cells following stressful stimuli.[36] Studies dealt with tissue inflammation demonstrate the upregulation of HSP70 which is of value in cellular stress,[37] and its expression is mostly linked to the degree of inflammation.[38]

HSP70 proteins have been implicated in the regulation of the immune responses and modulate inflammation through several mechanisms.[39]

Barbui *et al*.[15] clarified significantly higher plasma PTX-3 levels in PV than the median level of healthy controls. PTX-3 is produced locally by various cells, including monocytes, neutrophils, and ECs and released at the onset of inflammation.[40] During inflammation, studies attribute a crucial role for PTX-3 in the development of endothelial dysfunction, thereby signifying it as an acute-phase protein.[41] With this regard, a high-plasma level of the inflammatory molecule PTX-3 in the current study is speculated to be associated with endothelial dysfunction in PV patients.

PTX-3, by interacting with P-selectin following activation, it repositioned quickly to the cell surface, thereby help in the tethering and rolling of leukocytes and platelets on activated ECs.[42]

PTX-3 molecule also possesses proinflammatory and prothrombotic potential; it encourages TF in monocytes and ECs. Increased TF level (primary factor) of the coagulation cascade triggers the process of thrombus formation.[27] Furthermore, PTX-3 is well known to play a part in thrombosis and atherogenesis and associated with the occurrence of major thrombotic consequences in PV and essential thrombocythemia.[24-26]

Our results revealed a strong association between the levels of both HSP70 and PTX3 and JAK2^{V617F} allele burden (ratio between mutant and wild-type JAK2 in hematopoietic cells) which is in consistence with previous results.[15,43] This indicates that PTX3 signifies a state of chronic inflammation allied to disease severity and taking into account the JAK2^{V617F} mutation role in the pathogenesis of inflammation in MPNs.[44]

JAK2^{V617F} governs the marked activation of different cells, i.e., platelets, leukocytes, and ECs,[44,45] encourage the buildup of reactive oxygen species in the hematopoietic stem cell,[46] and arranging the structure of the inflammatory microenvironment of MPNs.[47] Substantially active *JAK2* signaling can precisely motivate the platelets and granulocytes activation and secondarily causes endothelial activation through consolidating platelet and leukocyte to ECs.[48,49]

Inflammation and hemostasis as a pathophysiologic process are correlated to and influence each other

forcefully in a bidirectional way. Inflammation guides the activation of the hemostatic system which, in turn, significantly motivates the inflammatory activity.[50] Furthermore, as a result of inflammation, there is an inequality between procoagulant and anticoagulant properties of the endothelium with consequent stimulation of the coagulation cascade. An additional attribute of inflammation is the interaction between leukocytes, ECs, and platelets. More crucially and irrespective of its causation, inflammation triggers endothelial activation.[51] Collectively, these processes would result in the loss of anticoagulant and vasodilatory physiological properties of the normal endothelium.

An interesting observation in PV is the relationship between higher HSP70 and PTX-3 concentrations. The latter has been considered an indicator of a systemic inflammatory response. It appears that systemic inflammation may be at least in part held responsible for augmented HSP70 concentration in PV patients, as implied by the noticeable positive link between HSP70 and PTX-3 concentrations. These data propose that HSP70 upregulation in PV patients compared to the controls could signify their inflammatory status.

Conclusion

Our study concludes an increased HSP70 and PTX-3 concentrations in patients with PV as compared with the controls. Clear relationships exist between PV and markers of inflammation. A thorough comprehending of why inflammation is prevalent in PV and how the host's immune system reacts to the neoplastic clone will likely disclose important understandings of PV disease pathogenesis and will also identify the novel therapeutic targets in this disease.

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Conflicts of interest

There are no conflicts of interest.

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