Resistant Anemia in CKD Patients Undergoing Hemodialysis (HD), What is the Role of Circulating Growth Arrest Specific Protein6 (Gas6)

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Abstract: Erythropoietin-Stimulating Agents (ESAs) resistance is one of the most common complications in anemia patients undergoing hemodialysis (HD). The poor response to ESAs therapy may be associated with increased mortality. Growth arrest-specific protein 6 (Gas6), a vitamin K-dependent protein, plays a role in cell growth, cell proliferation, anti-inflammation, and phagocytosis. Gas6 is expressed in some cell types, such as endothelial, smooth muscle, and bone marrow cells. Apart from its expression pattern, the biological role of Gas6 in hematopoiesis remains largely unknown. However, to date, the correlation between Gas6 and resistance remains unclear in HD patients. Therefore, the objective of this study was to investigate the relationship between circulating Gas6 levels and ESAs resistance in HD patients. The study was carried on 234 HD patients and 86 healthy individuals. The HD patients were divided into 2 groups: non-ESA-resistant patients and 36 ESA-resistant patients. Plasma levels of Gas6, high-sensitivity C-reactive protein (hs-CRP), and albumin were quantified. The obtained results revealed that, compared with non-ESA-resistant patients, EPO-resistant patients had elevated plasma concentrations of Gas6 plasma Gas6 (13.8 ± 3.17 vs. 6.59± 1.69 ng/mL, P <0.001), and hs-CRP (6.5±8.7vs. 1.1±0.5mg/L, P= 0.001). In contrast, compared with healthy subjects, HD patients had significantly lower levels of SI (59.85 ± 24.3 vs. 95.6 ± 28.9 µg/dL, P < 0.001), TIBC (265.3± 65.3 vs. 345.3 ± 98.6 µg/dL, P < 0.001), TSAT (24.5± 8.7 vs. 30.7 ± 14.3 %, P = 0.001), Hct (31.6 ± 2.4 vs. 41.6 ± 4.6 %, P < 0.001), and albumin (3.92 ± 0.32 vs. 4.78 ± 0.47 g/dL, P < 0.001). In ESA-resistant HD patients, plasma Gas6 levels were negatively correlated with albumin levels (r =−0.466, P <0.024). Moreover, in healthy controls, Gas6 levels were positively correlated with hs-CRP levels but negatively correlated with Hct and albumin levels. In conclusion, ours work showed that there was elevated circulating Gas6 levels in ESA-resistant HD patients. Compared with non-ESA-resistant HD patients, ESA-resistant HD patients had significantly increased concentrations of plasma Gas6, and hs-CRP. Moreover, circulating Gas6 levels were negatively correlated with albumin levels in ESA-resistant HD patients. Thus, ESA- resistance in HD patients is associated with inflammation and nutritional status. We recommend that circulating Gas6 levels might be used as a potential biomarker for ESA- resistance in HD patients.

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1. Introduction

Erythropoietin-Stimulating Agents (ESAs) resistance is one of the most common complications in anemia patients under-going hemodialysis (HD). The anemia of chronic kidney disease (CKD) is principally due to reduced renal erythro-poietin (EPO) production and, to a lesser degree, to shortened red cell survival. Anemia can develop well before the onset of uremic symptoms due to CKD. Many patients are relatively resistant to ESAs and require large doses. The poor response to ESAs therapy may be associated with increased mortality.[(1)] According to the European best practice guidelines (EBPG): The resistance to ESAs is defined as a failure to achieve target Hb levels (11– 12 g/dl) with doses lower than 300 IU/kg/ week of epoetin or 1.5 µg/kg/ week of darbopoietin-α.[(2)] KDIGO defines initial ESAs hyporesponsiveness as having no increase in hemoglobin concentration after the first month of appropriate weight-based dosing, acquired hypo-responsiveness as requiring two increases in ESAs doses up to 50 percent beyond the dose at which the patient had originally been stable. [(3)] Several factors have been described to promote ESAs resistance in HD patients as inflammation, malnutrition, aluminum toxicity, secondary hyperparathyroidism (sHPT), lower hemoglobin A1C (HbA1c), deplete iron stores and vitamin D deficiency have been found to be associated with ESA resistance. However, the combination of known risk factors enabling to stratify patients into responders and non-responders has not been investigated so far.[(4)]

Table (1): Show the risk factors, pathogeneses, and therapeutic modulation of erythropoietin resistance in pediatric and adult subjects with chronic kidney disease. [5]

Risk factors	Mechanism of ESA resistance	Therapeutic intervention
Uremic toxinsab	↓EPO synthesis/↓erythroid response	Longer effective dialysis
Oxidative stress a,b	Down regulation of HIF	Vit E and vit C
Inflammation a,b,c	Cytokines: IL-1, IL-6, TNF-α	Avoid sepsis and malnutrition
Iron deficiency a,c	Hemoglobin synthesis	Replenish iron/↓ blood loss
Hyperparathyroidisma, c	Vitamin D synergism (erythropoiesis)	Low P diet/ 1,25 OH vit D
Aluminum toxicity a,c	Aluminum bone disease	Avoid aluminum intake
Hemolysis a,c	Uremia/HbSS/G6PDD/AIHA	Uremic clearance/vit E and C
Drugs: angiotensin-modulating agents a,b	↓Erythroid ANG II receptors/↑endogenous EPO inhibitor, AcSDKP	↓ Dose of ACEi/ARB

Abbreviations: ACEi, angiotensin-converting enzyme inhibitor; AcSDKP, *N*-acetyl-seryl-aspartyl-lysyl-proline; AIHA, autoimmune hemolytic anemia; ANG, angiotensin; ARB, angiotensin receptor blocker; EPO, erythropoietin; ESA, EPO-stimulating agent; G6PDD, glucose 6-phosphatase deficiency; HbSS, hemoglobinopathies; HD, hemodialysis; HIF, hypoxic-inducible factor; IL, interleukin; TNF-α, tumor necrosis factor-α; vit, vitamin.

A Adult data; applicable to both populations.

B Experimental data; applicable to both populations.

C Pediatric and adult data.

Growth arrest-specific protein 6 (Gas6), a vitamin K-dependent protein, plays a role in cell growth, cell proliferation, anti-inflammation, and phagocytosis. Gas6 is expressed in some cell types, such as endothelial, smooth muscle, and bone marrow cells. It was recently reported that elevated circulating Gas6 levels are correlated with disease activity such as systemic inflammation, acute coronary syndrome, acute pancreatitis, and Systemic lupus erythe-matosus. In chronic kidney disease patients, Gas6 levels were associated with renal disease and inversely related to renal function, suggesting that Gas6 levels may serve as a biomarker for disease stages and its complications.[6] Apart from its expression pattern, the biological role of Gas6 in hematopoiesis remains largely unknown. However, to date, the correlation between Gas6 and resistance remains unclear in HD patients. Therefore, the objective of this study was to investigate the correlation between circulating Gas6 levels and ESAs resistance in HD patients.

Patients and Methods:

This study was performed on 86 healthy subjects were randomly recruited from an unselected population, as the control group and 234 HD patients that were recruited from Internal Medicine Department, Tanta University Hospital between April 2015 and February 2016, this study was approved by the human research ethics committee of the hospital, and informed consent was obtained from each patient. Patients with sepsis, iron deficiency, folate deficiency, vitamin B deficiency, bone marrow diseases, CAD, and hematopoietic diseases were excluded from this study. The HD patients were further divided into two groups: ESAs-resistant patients were defined as those receiving a high dose of EPO ≥ 40,000 U/month for a minimum of 6 months and with hematocrit (Hct) levels<33 %, and non- ESAs -resistant patients were defined as those receiving a low dose of EPO < 40,000 U/month fora minimum of 6 months and with Hct levels >33 %. All patients underwent regular HD 3 times/week, after enrollment, fasting morning serum samples were obtained from the subjects and were stored at −80°C until analysis. Biochemical assessments were performed, including measurement of high-sensitivity C-reactive protein (hs-CRP), blood urea nitrogen (BUN), albumin, phosphate, calcium, intact parathyroid hormone (i-PTH), Hct, ferritin, serum iron (SI), transferring saturation (TSAT), total iron-binding capacity (TIBC), and CBC. Measurement of growth arrest‐specific protein: Plasma Gas6 concentrations were determined using commercial enzyme immunoassay kits (R & D Systems, Minneapolis, MN), according to the manufacturer's instructions.[6]

Statistics:

Statistical presentation and analysis of the present study was conducted, using the mean, standard deviation and chi-square test by SPSS V.20.

***1.* Mean value** $\left(\bar{X}\right)$ **:** The sum of all observations divided by the number of observation:

$$\left(\bar{X}\right) = \frac{\Sigma x}{n}$$

Where Σ = sum & n = number of observations.

***2.* Standard Deviation [SD]:**

It measures the degree of scatter of individual varieties around their mean:

$$SD = \sqrt{\frac{\Sigma \left| x - \bar{x} \right|^{-2}}{n - 1}}$$

***3.* Standard student "t test",** test of significance of the difference between two means:

$$t = \frac{\bar{X} - \bar{X}_2}{\sqrt{\frac{(SD_1)^2}{n_1} + \frac{(SD_2)^2}{n_2}}}$$

The calculated "t" was compared with tabulated one at different levels of significance at the degree of freedom (DF):

DF = (d + n2) -2 Where:

$\bar{X}$ = The mean value of group L

$\bar{X}_2$ = The mean value of group II.

SD1 = The standard deviation of group I.

SD2 = The standard deviation of group II.

n1= The number of observations of group L

n2= The number of observations of group II.

***4.* Linear Correlation Coefficient [r]:**

$$r = \frac{\Sigma (X - \bar{X})(y - \bar{y})}{\sqrt{\{\Sigma (X - \bar{x})2\}\{\Sigma (y - \bar{y})2\}}}$$

Where:

X= Independent variable.

Y= Dependent variable

Results:

Patient characteristics with comparison of blood biochemistry data between the patients and control groups:

As shown in table (1); The 234 HD patients comprised 86 male and 148 female patients with an average age of 42.2 ± 13.3 years and an average HD period of 81.0 ± 39.9 months. The HD patients were further divided into 2 groups: (1) 58 EPO-resistant HD patients receiving a high dose of EPO ≥ 40,000 U/month for a minimum of 6 months and with Hct levels <33 %; and (2) 176 non-EPO-resistant HD patients comprising receiving a low dose of EPO < 40,000 U/month for a minimum of 6 months and with Hct levels >33 %. Biochemical characteristics between control and hemodialysis subjects are shown in Table 1 that showing that, HD patients had significantly higher concentrations of serum ferritin (428.7±275.6vs. 163.8±47.8 ng/mL, P < 0.001), i-PTH (439.8± 258.4vs. 36.4 ± 9.8 pg/mL, P < 0.001), phosphate (5.34± 1.36 vs. 3.41± 0.53 mg/dL, P <0.001), plasma Gas6 (13.8±3.17 vs. 6.59 ± 1.69 ng/mL, P < 0.001), and hs-CRP (6.5± 8.7vs. 1.1±0.5 mg/L, P = 0.001). In contrast, compared with healthy subjects, HD patients had significantly lower levels of SI (59.85± 24.3 vs. 95.6± 28.9µg/dL, P < 0.001), TIBC(265.3± 65.3 vs. 345.3±98.6 µg/dL, P < 0.001), TSAT(24.5± 8.7 vs. 30.7± 14.3 %, P = 0.001), Hct (31.6± 2.4 vs. 41.6± 4.6 %, P < 0.001), and albumin (3.92± 0.32 vs.4.78± 0.47g/dL, P < 0.001).

Measurement of Gas6 and hs-CRP levels in ESA - resistant hemodialysis patients compared with non-ESA -resistant patients:

Table (2): Biochemical characteristic of study subjects with comparison of blood biochemistry data between the patients and control groups.

Item	Patients (n=234)	Control (n=60)	P value
Ferritin (ng/ml)	428.7 ± 275.6	163.8 ± 47.8	<0.001*
SI (µg/dl)	59.85 ± 24.3	95.6 ± 28.9	<0.001*
TSAT (%)	24.5 ± 8.7	30.7 ± 14.3	<0.001*
TIBC (µg/dl)	265.3 ± 65.3	345.3 ± 98.6	<0.001*
i-PTH (pg/ml)	439.8 ± 258.4	36.8 ± 9.8	<0.001*
Hematocrit (%)	31.6 ± 2.4	41.6 ± 4.5	<0.001*
Albumin (g/dl)	3.92 ± 0.32	4.78 ± 0.47	<0.001*
Phosphate (mg/dl)	5.34 ± 1.36	3.41 ± 0.53	<0.001*
Calcium (mg/dl)	9.29 ± 0.78	9.12 ± 0.57	0.115
Gas6 (ng/ml)	13.8 ± 3.17	6.59 ± 1.69	<0.001*
hs-CRP (mg/l)	6.5 ± 8.7	1.1 ± 0.5	<0.001*

<0.001* (statically significant) Data are mean ± SD, EPO erythropoietin, SI serum iron, TSAT transferring saturation, TIBC total iron binding capacity, i-PTH intact parathyroid hormone, Gas6 growth arrest-specific, Hs-CRP high sensitivity C-reactive protein, RBC red blood cell

Table (3): A comparison of blood biochemistry data between the EPO-resistant and non-EPO-resistant HD patients.

Item	Non-EPO resistance (n=176)	EPO resistance (n=58)	P value
Ferritin (ng/ml)	418.4 ± 330.8	428.3 ± 310.0	0.841
SI (µg/dl)	61.3 ± 22.3	57.55 ± 24.8	0.281
TSAT (%)	25.1 ± 11.2	23.9 ± 10.0	0.469
TIBC (µg/dl)	267.7 ± 58.0	262.9 ± 64.0	0.579
i-PTH (pg/ml)	427.9 ± 360.0	415.7 ± 396.0	0.827
Hematocrit (%)	32.5 ± 2.4	30.7 ± 2.7	<0.001*
Albumin (g/dl)	3.94 ± 0.3	3.9 ± 0.4	0.421
Phosphate (mg/dl)	5.18 ± 1.5	5.5 ± 1.5	0.160
Calcium (mg/dl)	9.39 ± 0.8	9.19 ± 0.9	0.111
Gas6 (ng/ml)	13.1 ± 3.2	14.5 ± 3.3	0.005*
hs-CRP (mg/l)	3.5 ± 2.6	9.5 ± 3.6	<0.001*

EPO erythropoietin, SI serum iron, TSAT transferring saturation, TIBC total iron binding capacity, i-PTH intact parathyroid hormone, Gas6 growth arrest-specific6, Hs-CRP high sensitivity C-reactive protein, RBC red blood cell

Table (4): A comparison of clinical variables associated with Gas6 levels (Correlation between Gas6 and albumin levels in ESA-resistant and non- ESA -resistant hemodialysis patients).

With	Gas6 (ng/ml)					
	Control		Non-EPO resistance		EPO resistance	
	r	P	r	p	r	p
Ferritin (ng/ml)	0.085	0.758	- 0.158	0.198	- 0.214	0.125
SI (µg/dl)	- 0.385	0.086	- 0.079	0.658	- 0.185	0.214
TSAT (%)	- 0.185	0.185	- 0.135	0.524	- 0.210	0.154
TIBC (µg/dl)	- 0.064	0.812	0.081	0.647	0.095	0.785
i-PTH (pg/ml)	0.095	0.634	0.189	0.174	0.086	0.793
Hematocrit (%)	- 0.421	0.031*	0.167	0.158	- 0.201	0.167
Albumin (g/dl)	- 0.552	0.012*	- 0.124	0.587	- 0.466	0.024*
Phosphate (mg/dl)	0.091	0.701	- 0.116	0.635	- 0.254	0.108
Calcium (mg/dl)	- 0.102	0.612	- 0.014	0.932	0.127	0.574
hs-CRP (mg/l)	0.126	0.582	- 0.021	0.914	0.058	0.841

Gas6 growth arrest-specific 6, EPO erythropoietin, SI serum iron, TSAT transferring saturation, TIBC total iron-binding capacity, i-PTH intact parathyroid hormone, Hcthematocrit, Hs-CRP high sensitivity C-reactive protein, EPO erythropoietin

As shown in table (2); (there was elevated growth arrest‑specific protein 6 and hs-CRP levels in ESA-resistant hemodialysis patients on comparison with ESA-resistant hemodialysis patients). By Compared with non-EPO-resistant patients, ESA -resistant patients had elevated concentrations of plasma Gas6 (14.5±3.3 vs. 13.1± 3.2 ng/mL, P = 0.005), and hs-CRP (9.5±3.6 vs. 3.5 ±2.6 mg/L, P = 0.001). However, non- ESA -resistant patients had higher Hct concentrations than ESA-resistant patients.

Correlation between Gas6 and albumin levels in ESA-resistant and non- ESA -resistant hemodialysis patients:

A comparison of clinical variables associated with Gas6 levels is presented in Table 3. In ESA-resistant HD patients, plasma Gas6 levels were negatively correlated with albumin levels ($r = -0.466$, $P < 0.024$). Moreover, in healthy controls, Gas6 levels were positively correlated with hs-CRP levels but negatively correlated with Hct and albumin levels.

Discussion and conclusions

Many crucial questions about optimal anemia control among HD patients are not adequately answered yet. Relatively large percentage of anemic patents who receive therapy with ESA are either hyporesponsive or resistant to ESA. The poor response to ESA therapy may be associated with increased mortality. Growth arrest-specific protein 6 (Gas6) works synergistically with erythropoietin (EPO) to increase the proliferation and maturation of erythro-blasts. However, the role of Gas 6 levels on EPO resistance in hemodialysis (HD) patients remains unclear.[7,8] In our present study, the results showed that elevated circulating Gas6 levels in ESA-resistant HD patients compared with non-ESA-resistant HD patients, ESA-resistant HD patients had significantly increased concentrations of plasma Gas6, and hs-CRP. Moreover, circulating Gas6 levels were negatively correlated with albumin levels in EPO-resistant HD patients, Our findings are supported with the findings of **Miao‐et al., 2016** [8], who found that elevated circulating Gas6 levels in EPO-resistant HD patients. Compared with non-EPO-resistant HD patients, EPO resistant HD patients had significantly increased concentrations of plasma Gas6, and hs-CRP. Moreover, they reported that circulating Gas6 levels might be used as a potential biomarker for EPO resistance in HD patients.

Another supporting reports came from Korea as ***Yanagita et al., 2002 and Abe et al., 2011***[9,10] stated that Gas6 enhanced EPO receptor signaling stimulating hemoglobin production, thus playing the role of a hemoglobin regulator also these studies EPO resistance in HD patients were linked to circulating Gas6 levels. This indicates that EPO resistance is linked to systemic inflammation in HD patients and Gas6 was proposed to be a potential regulator of immune response in the development of systemic inflammation.

Our study also revealed that circulating Gas6 levels were negatively correlated with albumin levels ($r = -0.466$, $P < 0.024$) in ESA resistant HD patients. Our findings are consistent with the results of recent studies [8,11] that showing that plasma Gas6 levels were negatively correlated with serum albumin levels patients, implying that EPO resistance could be linked to nutritional status. Similar findings were recently reported by ***Lee et al. (2012), and Hurtado and de Frutos, (2010)*** [6,12]**,** whom found that higher Gas6 levels were associated with low albumin levels in chronic kidney disease patients, suggesting a possible connection between malnutrition and inflammation associated with worsening renal function. Moreover, resistance to EPO treatment was associated with low albumin levels in HD patients.

In conclusion, ours work showed that there was elevated circulating Gas6 levels in ESA-resistant HD patients. Compared with non-ESA-resistant HD patients, ESA-resistant HD patients had significantly increased concentrations of plasma Gas6, and hs-CRP. Moreover, circulating Gas6 levels were negatively correlated with albumin levels in ESA-resistant HD patients. Thus, ESA-resistance in HD patients is associated with inflammation and nutritional status.

We recommend that circulating Gas6 levels might be used as a potential biomarker for ESA-resistance in HD patients. Also a promising experimental study by Anne et al., 2008 [13] reported that the protein product of growth arrest–specific gene 6 (Gas6) is important for cell survival across several cell types also when mice with acute anemia were treated with Gas6, the protein normalized hematocrit levels without causing undesired erythrocytosis. Also in a mouse model of chronic anemia caused by insufficient Epo production, Gas6 synergized with Epo in restoring hematocrit levels. These findings may have implications for the treatment of patients with anemia who fail to adequately respond to Epo. So according to these findings we recommend further investigations of the therapeutic potential of Gas6 for the treatment of patients who are hyporesponsive or resistant to ESA.

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