Clinical Significance of Serum Biomarkers: Vascular Endothelial Growth Factor-A and Chemokine (C-X-C motif) Ligand 13 in Egyptian Multiple Myeloma Patients

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Abstract- Multiple myeloma (MM) is a human B-cell neoplasia arising from malignant plasma cells. Vascular endothelial growth factor (VEGF) is among growth factors essential for angiogenesis in MM. However, chemokine (C-X-C motif) ligand 13 (CXCL13) allows the chemotaxis of mature B cells expressing its receptor CXCR5.CXCL13-CXCR5 interactions are involved in MM progression. This study aimed at investigating 2 serum biomarkers; VEGF-A and CXCL13 levels using enzyme linked immunosorbent assays (ELISA) in 48 Egyptian myeloma patients as well as correlation with different clinic-pathological features, survival and therapy response. VEGF-A and CXCL13 levels were significantly higher in MM cases in comparison to control group (*P*=0.04* and 0.01*, respectively). An indirect proportional relation between VEGF-A and CXCL13 levels in myeloma patients was found (r= -0.27, *P*=0.22). Alb/creat ratio change showed indirect proportional relation with VEGF-A (r= -0.446, *P*=0.043*). Patients obtained complete remission (CR)had insignificantly lower VEGF-A and higher CXCL13 levels compared to other patients, *P*=0.2 and 0.7, respectively. In conclusion, production of variety of growth factors and cytokines such as VEGF-A and CXCL13 was higher in MM patients. However, our experiment has to be done on larger sample size and extended period of follow up to validate the participation of the VEGF-A and CXCL13 in disease progression and clinical outcome.

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Introduction

Multiple myeloma (MM) is a human B-cell neoplasia arising from malignant plasma cells having incidence of ~1% of all neoplasias and >10% of all hematological malignancies with a median survival of 3-5 years despite all available treatment approaches (1,2). In 2018, there were approximately 871 new Egyptian MM cases were detected, presenting 0.68% of all new cancer patients while, related deaths representing~ 783 deaths (0.92%) of all deaths (3). Renal injuries are a common complication resulting from deposition of nephrotoxic monoclonal Ig or may be independent of paraprotein deposition (4). Physiological production of new blood vessel from present vessels happens throughout normal growth and tissue healing with abnormal increase in angiogenesis in tumor development and spread had been noticed and linked to poor prognosis in hematological malignancies such as MM (5). Increased bone marrow (BM) micro vessel density in MM patients is a strong prognostic factor (6). Vascular endothelial growth factor (VEGF) is among growth factors needed for angiogenesis in MM which encourage vascular permeability, endothelial cells (EC) migration, proliferation and survival via activation of the RAS/RAF/ERK/MAPK pathways (7). Mutation of

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iKRAS and NRAS genes have a crucial effect on pathogenesis, progression and prognosis of MM which can be determined by various molecular approaches (8). The VEGF family formed of 5 members: VEGF-A, placenta growth factor (PGF), VEGF-B, VEGF-C and VEGF-D (9). Angiogenesis is used as a crucial driver and target for myeloma treatment. Many approaches have been followed such as drugs targeting both MM and neovessels such as the immunomodulatory drugs (IMiDs) and the proteasome inhibitors along with drugs that interfere with specific EC functions through changing cell signaling pathways activating angiogenesis such as bevacizumab/Avastin (10). Chemokine (C-X-C motif) ligand 13 (CXCL13), is homeostatic chemokine that allows chemotaxis of mature B cells expressing its receptor CXCR5 (11). CXCL13-CXCR5 interactions are implicated in malignant cell homing, adhesion, signal transduction, and calcium flux, that result in MM progression (12). This study aimed at investigating 2 serum biomarkers: VEGF-A and CXCL13 levels in Egyptian myeloma patients as well as correlation with different clinic-pathological features, overall survival, disease-free survival & response to therapy.

Materials and Methods

Study population

Our study included 48 MM patients fulfilling criteria according to (IMWG) for symptomatic MM. Patients were recruited from November 2018 to May 2019 after getting approval of research ethical committee of our clinical oncology dept. Patients having renal affection due to diseases other than MM as diabetes and hypertension were excluded from our study together with patients having monoclonal gammopathy of undetermined significance (MGUS), POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes) syndrome, smoldering MM and solitary plasmacytoma . Control group included 20 age and sex matched healthy population. For patients and controls, 2 ml serum blood samples were collected.

Quantitative detection of serum VEGF-A using enzyme linked immunosorbent assays (ELISA)

Serum VEGF-A levels were measured by human VEGF-A Platinum ELISA kit provided by Invitrogen; Thermo Fisher Co., USA (Cat. No.: BMS277-2) following the manufacturer's protocol that had a detection range of 15.6-1,000 pg/mL and sensitivity of 7.9 pg/mL. Briefly, Microwell strips were washed twice with approximately 400 µL wash buffer per well with thorough aspiration of microwell contents between washes. One hundred µl of standard or blank, and 50 µl of samples (sera from patients and healthy controls) were added in different wells of a 96- well plate, covered by adhesive film and incubated at room temperature (18° to 25° C) for 2 hours on a microplate shaker set at 400 rpm. The adhesive film was removed, and wells were emptied. Microwell strips were washed 6 times. A 100 µL of Biotin-Conjugate was added to all wells. Wells were covered by adhesive film and incubated at room temperature (18° to 25° C) for 1 hour on a microplate shaker set 400 rpm. After washing 6 times, a 100 µL of diluted Streptavidin-HRP was added to all wells, including the blank wells. Wells were covered by adhesive film and incubated at room temperature (18°to 25° C) for 1 hour on a microplate shaker set 400rpm. Again, after washing for 6 times, a 100 µL of TMB Substrate Solution was added to the wells. The microwell strips were incubated at room temperature (18°-25° C) for about 30 min. The enzyme reaction was stopped by quickly adding 100 µL of stop solution into each well and the plate was read in an ELI SA reader at 450 nm.

Quantitative detection of serum CXCL13 using enzyme linked immunosorbent assays (ELISA)

Serum CXCL13 levels were measured by human CXCL13 EIAab ELISA kit provided by Wuhan EIAab Science Co., China (Cat. No.: E1601h) following the manufacturer's instructions with detection range of 7.8-500 pg/mL and sensitivity of 3.2 pg/mL. Briefly, one hundred µl of standard or blank, samples (sera from patients and healthy controls) were added in different wells of a 96- well plate, covered by plate sealer and incubated at 37° C for 2 hours. The sealer was removed and the wells were emptied. A 100 µL of detection reagent A working solution was added to all wells. Wells were covered by plate sealer and incubated at 37° C for 1 hour. After washing 3 times, a 100 µL of detection reagent B working solution was added to all wells. Wells were covered by plate sealer and incubated at 37° C for 1 hour. Again, after washing for 5 times, a 90 µL of Substrate Solution was added to the wells. The microwell strips were incubated at 37° C for about 10-20 min. The enzyme reaction was stopped by adding 50 µL of stop solution into each well and the plate was read in ELISA microplate reader at 450 nm.

Data analysis

Description of data was done in the form of mean±standard deviation (±SD), median and range, or frequencies and percentages when needed. Numerical data were tested using the Shapiro Wilk test. Numerical variables were compared using Mann Whitney *U* test for independent samples in comparing 2 groups and Kruskal Wallis test in comparing more than 2 groups. Comparison of categorical data was done throughChi-square (χ^2) test and exact test was utilized instead if the expected frequency is <5. Correlation between various variables was done through Spearman rank correlation equation. Survival analysis was done through Kaplan Maier statistics calculating the mean and median survival time for each group with their 95%CI and the corresponding survival graphs. Two-sided $P<0.05$ was known as statistically significant. All statistical calculations were done using IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Windows.

Results

Twenty-five males and 23 females were enrolled in our study with a male to female ratio 1.09 and ages range between 36 and 72 years with mean±SD of 53.7±8.95 years and median of 54 years.

VEGF-A levels in controls and MM patients

Regarding control group, VEGF-A level has a range of 60.91 to 727.6 pg/ml with a mean±SD of 316.3±231.67 pg/ml and a median of 279.6 pg/ml. However, in MM patients, it has a range of 50 to 1800 pg/ml with a mean±SD of 625.62±466.42 pg/ml and a median of 500 pg/ml, with statistically significant difference between the two groups (P=0.04*). MM patients with VEGF-A level less than cut off value that was the mean level in the control population (316.3) were classified as "*low VEGF-A level"*, while those with level higher than (316.3) were recognized as "*high VEGF-A level"*. Sixteen patients (33.3%) had a low VEGF-A level, while, 32 patients (66.7%) had high VEGF-A level. Characteristics of myeloma patients according to low and high VEGF status were shown in table 1. Regarding serum calcium level, 4/14 patients (28.6%) had calcium level≥12 mg/dl and low VEGF-A level compared to only one patient (1/25; 4%) who had calcium level≥12 and had high VEGF-A level, with statistically significant difference among the 2 groups (P=0.04*). Significant higher Alb/creat ratio change was noticed in patients who had low VEGF-A level compared to high level (P=0.013*). As regards Alb/creat ratio improvement, all patients who had low VEGF-A level showed no improvement compared to 5/15 patients (33.3%) who had high VEGF-A level with statistically significant difference among the 2 groups (P=0.01*). The relation between serum VEGF-A levels and different MM patients' clinic-pathological features was described in table 2. Again, regarding Alb/creat ratio improvement, median VEGF-A level was significantly higher in patients who improved.

CXCL13 levels in controls and MM patients

In the control group, CXCL13 level ranged between 4.44 and 14.22 pg/ml with a mean±SD of9.12±4.06pg/ml and a median of 8.9 pg/ml. CXCL13 level was performed for only 22 patients of our studied MM patients' group, it ranged between 70.28 and 222.4 pg/ml with a mean±SD of137.06±42.83 pg/ml and a median value of 134.6 pg/ml, with statistically significant difference among the 2 groups (P=0.01*). MM patients with CXCL13 level less than cut off value that was the mean level in the control group (9.12) were classified as "*low CXCL13 level"*, while those with level higher than (9.12) were recognized as "*high CXCL13 level"*. All 22 MM patients had high CXCL13 level with patients characteristics described in table 1. The relationship between serum CXCL13 levels with different MM patients' clinic-pathological features was described in table 3 with no significant difference was found.

Correlation between VEGF-A and CXCL13 and overall survival (OS) and disease-free survival (DFS) in MM patients

MM patients were followed in accordance with available clinical data, the overall survival rate (OS)and disease-free survival rate (DFS) were calculated. Different MM treatment modalities in relation to OS and DFS were shown in table 4. Patients who received Velcade® (bortezomib)-Thalidomide-dexamethasone, showed the highest median OS and DFS (69.38 and 61.5 months, respectively). Patients with low VEGF-A level had higher mean OS and DFS rates compared to patients who had high VEGF-A level (98.53 and 74.6 vs 44.88 and 18.2, respectively); however, this is not reached statistically significant difference (P=0.89 and 0.24, respectively). Kaplan Maier analysis for OS and DFS between the VEGF-A & CXCL13 levels was described in table 5 and figures 1-4.

Table 1. Characteristics of MM patients according to their VEGF-A and CXCL13 status

Items		No. of patients (%)		*P*	No. of patients (%)		*P*
		Low VEGF-A (n=16, 33.3%)	High VEGF-A (n=32, 66.7%)		Low CXCL13 (n=0, 0%)	High CXCL13 (n=22, 100%)	
Age (years)	> 65	0	3/32 (9.4%)	0.54	-	1/22 (4.5%)	-
	≤ 65	16/16 (100%)	29/32 (90.6%)			21/22 (95.5%)	
Gender	Male	7/16 (43.8%)	18/32 (56.3%)	0.5	-	12/22 (54.5%)	-
	Female	9/16 (56.3%)	14/32 (43.8%)		-	10/22 (45.5%)	
Serum M-protein	IgG Kappa	8/14 (57.1%)	14/28 (50%)	0.3	-	11/16 (68.8%)	-
	IgG Lambda	2/14 (14.3%)	8/28 (28.6%)			2/16 (12.5%)	
	IgA Kappa	4/14 (28.6%)	2/28 (7.1%)			2/16 (12.5%)	
	IgA Lambda	0	2/28 (7.1%)			0	
	Light chains	0	2/28 (7.1%)			1/16 (6.3%)	
ISS stage	I	2/8 (25%)	3/13 (23.0%)	0.96	-	3/15 (20%)	-
	II	2/8 (25%)	4/13 (30.8%)			6/15 (40%)	
	III	4/8 (50%)	6/13 (46.2%)			6/15 (40%)	
β2m (mg/L)	Less than 3.5	3/11 (27.3%)	6/18 (33.3%)	1	-	4/15 (26.7%)	-
	More than or equal 3.5	8/11 (72.7%)	12/18 (66.7%)			11/15 (73.3%)	
Albumin (g/L)	Less than 3.5	9/13 (69.2%)	18/29 (62.1%)	0.74	-	8/16 (50%)	-
	More than or equal 3.5	4/13 (30.8%)	11/29 (37.9%)			8/16 (50%)	
Hemoglobin (g/dl)	Less than or equal 10	9/14 (64.3%)	18/30 (60%)	1	-	7/18 (38.9%)	-
	More than 10	5/14 (35.7%)	12/30 (40%)			11/18 (61.1%)	
Platelets ($x10^9$/L)	Less than or equal 100	1/14 (7.1%)	4/30 (13.3%)	1	-	1/18 (5.6%)	-
	More than 100	13/14 (92.9%)	26/30 (86.7%)			17/18 (94.4%)	
Serum calcium (mg/dL)	More than or equal 12	4/14 (28.6%)	1/25 (4%)	0.04*	-	2/13 (15.4%)	-
	Less than 12	10/14 (71.4%)	24/25 (96%)			11/13 (84.6%)	
Serum creatinine (mg/dl)-baseline	More than or equal 2	4/14 (28.6%)	8/30 (26.7%)	1	-	18/18 (100%)	-
	Less than 2	10/14 (71.4%)	22/30 (73.3%)				
Alb/creat ratio (mg/g)-baseline	More than or equal 300	1/12 (8.3%)	6/18 (33.3%)	0.19	-	1/4 (25%)	-
	Less than 300	11/12 (91.7%)	12/18 (66.7%)			3/4 (75%)	
Serum creatinine (3 ms)	More than or equal 2	3/12 (25%)	5/18 (27.8%)	1	-	4/4 (100%)	-
	Less than 2	9/12 (75%)	13/18 (72.2%)				
Alb/creat ratio (3 ms)	More than or equal 300	0	2/15 (13.3%)	1	-	1/4 (25%)	-
	Less than 300	6/6 (100%)	13/15 (86.7%)			3/4 (75%)	
Creatinine change	Range	-2.6-0.5	-6-0.7	0.89	-	-0.1-0.5	-
	mean±SD	-0.63±1.07	-0.81±1.87			0.168±0.26	
	median	-0.3	-0.3			0.14	
Alb/creat ratio change	Range	4-4,530	-8.7-315	0.013*	-	87-4.530	
	mean±SD	957.8±1.77	10.33±125.47			1,425±2,089	
	median	217.3	-39.86			541.5	
Creatinine improvement	Not improved	9/12 (75%)	12/18 (66.7%)	0.7	-	4/4 (100%)	-
	Improved	3/12 (25%)	6/18 (33.3%)				
Alb/creatratio improvement	Not improved	6/6 (100%)	5/15 (33.3%)	0.01*		4/4 (100%)	-
	Improved	0	10/15 (66.7%)				
Osteolytic lesions	Yes	6/14 (42.9%)	8/28 (28.6%)	0.49	-	1/16 (6.3%)	-
	No	8/14 (57.1%)	20/28 (71.4%)			15/16 (93.8%)	
Response to MM treatment	CR	3/8 (37.5%)	1/13 (7.7%)	0.13	-	2/15 (13.3%)	-
	VGPR	0	2/13 (15.4%)			2/15 (13.3%)	
	PR	2/8 (25%)	4/13 (30.8%)			5/15 (33.3%)	
	PD	3/8 (37.5%)	2/13 (15.4%)			2/15 (13.3%)	
	ST	0	4/13 (30.8%)			4/15 (26.7%)	

*: Significant at $P \leq 0.05$

β2m, beta-2-microglobulin; ISS, International Staging System; Alb/creat, Albumin/creatinine; CR, complete remission; VGPR, very good partial response; PR, partial response; PD, progressive disease; ST, stationary disease

Table 2. Characteristics of MM patients according to their VEGF-A status

Items		VEGF-A*(pg/ml)*			*P*
		Range	Mean±SD	Median	
Age (years)	**> 65**	350-1,000	698 ± 327.43	744	0.64
	< 65	50-1,800	624.7 ± 472.1	500	
Gender	**Male**	50-1,500	629.52 ± 399.04	520	0.7
	Female	56-1,800	628.9 ± 531.53	456	
Serum M-protein	**IgG Kappa**	50-1,800	629.5 ± 526.77	428	0.27
	IgG Lambda	100-1,440	662.2 ± 401.82	624	
	IgA Kappa	112-744	352.67 ± 233.43	275	
	IgA Lambda	1,000-1,180	1,090 ± 127.28	1,090	
ISS stage	**I**	106-744	426.8 ± 254.22	456	0.68
	II	100-1,400	686.67 ± 536.46	690	
	III	50-1,500	763.2 ± 605.46	675	
β_2m (mg/L)	**Less than 3.5**	104-1,800	709.56 ± 553.42	670	0.94
	More than or equal 3.5	50-1,500	669.3 ± 520.7	460	
Albumin (g/L)	**Less than 3.5**	56-1,800	697.6 ± 515.85	670	0.56
	More than or equal 3.5	50-1,480	564.07 ±376.13	500	
Hemoglobin (g/dl)	**Less than or equal 10**	56-1,480	585.96 ± 424.544	510	0.33
	More than 10	50-1,800	748.59 ± 523.91	670	
Platelets (x10^9/L)	**Less than or equal 100**	300-744	498.4 ± 176.99	510	0.7
	More than 100	50-1,800	668.08 ± 489.38	520	
Serum calcium (mg/dL)	**More than or equal 12**	100-1,000	336 ± 380.1	180	0.08
	Less than 12	50-1,800	683.26 ± 488.27	549	
Serum creatinine (mg/dl)-baseline	**More than or equal 2**	100-1,180	621.92 ± 389.16	590	0.99
	Less than 2	50-1,800	658.9 ± 497.6	495	
Alb/creat ratio (mg/g)-baseline	**More than or equal 300**	112-1,500	682.86 ± 482.19	510	0.34
	Less than 300	50-1,800	548.13 ± 475.5	360	
Serum creatinine (3 ms)	**More than or equal 2**	100-1,000	509.38 ± 403.96	430	0.73
	Less than 2	50-1,800	605.09 ± 500.99	490	
Alb/creat ratio (3 ms)	**More than or equal 300**	400-500	450 ± 70.71	450	0.47
	Less than 300	50-1,800	723.1 ± 491.07	670	
Creatinine improvement	**Not improved**	50-1,800	579.33 ± 526.58	400	0.56
	Improved	106-1,000	580.11 ± 339.29	578	
Alb/creat ratio improvement	**Not improved**	50-1,440	483.36 ± 419.33	300	0.008*
	Improved	510-1,800	932.2 ± 430.38	872	
Osteolytic lesions	**Yes**	56-1,800	574.79 ±501.98	435	0.39
	No	50-1,500	667.86 ± 465.27	515	
Response to MM treatment	**CR**	200-1,500	577.5 ± 629.19	260	0.21
	VGPR	1,200-1,480	1,340 ± 197.99	1,340	
	PR	50-1,440	591.33 ± 541.58	479	
	PD	106-744	335.6 ± 269.13	265	
	ST	350-1,400	937.5 ± 434.69	1,000	

*: Significant at $P \leq 0.05$

β2m, beta-2-microglobulin; ISS, International Staging System; Alb/creat, Albumin/creatinine; CR, complete remission; VGPR, very good partial response; PR, partial response; PD, progressive disease; ST, stationary disease

Table 3. Characteristics of MM patients according to their CXCL13 status

Items		CXCL13*(pg/ml)*			*P*
		Range	Mean±SD	Median	
Age (years)	**> 65**	194	194	194	0.18
	< 65	70-222	134.3 ± 41.89	134.6	
Gender	Male	101-222	152.78 ± 36.79	148.85	0.075
	Female	70-194	118.19 ± 43.61	102.25	
Serum M-protein	**IgG Kappa**	101-222	147.47 ± 40.66	134.6	0.07
	IgG Lambda	70-73	71.65 ± 1.94	71.65	
	IgA Kappa	80-165	122.29 ± 59.7	122.29	
	IgA Lambda	-	-	-	
	Light chains	99	98.5	98.5	
ISS stage	**I**	80-165	115.7 ± 43.72	102.6	0.95
	II	70-183	127.13 ± 46.94	134.6	
	III	99-194	129.37 ± 38.31	113.8	
β2m (mg/L)	**Less than 3.5**	73-165	125.98 ± 45.7	133.2	0.89
	More than or equal 3.5	70-222	138.32 ± 46.45	134.6	
Albumin (g/L)	**Less than 3.5**	70-194	124.6 ± 45.19	118.25	0.64
	More than or equal 3.5	80-222	137.51 ± 47.41	130.15	
Hemoglobin (g/dl)	**Less than or equal 10**	80-222	145.01 ± 55.12	134.6	0.53
	More than 10	70-167	128.19 ± 36.33	134.6	
Platelets ($x10^9$/L)	**Less than or equal 100**	80	80.07	80.07	0.2
	More than 100	70-222	137.95 ± 42.9	134.6	
Serum calcium (mg/dL)	**More than or equal 12**	135-164	149.2 ± 20.65	149.2	0.49
	Less than 12	70-222	131.64 ± 48.08	125.7	
Serum creatinine (mg/dl)-baseline	**More than or equal 2**	-	-	-	-
	Less than 2	70-222	134.73 ± 43.82	134.6	
Alb/creat ratio (mg/g)-baseline	**More than or equal 300**	222	222	125.7	0.18
	Less than 300	70-135	110.2 ± 34.85		
Serum creatinine (3 ms)	**More than or equal 2**	-	-	-	-
	Less than 2	70-222	138.25 ± 62.91	130.2	
Alb/creat ratio (3 ms)	**More than or equal 300**	222	222	222	0.18
	Less than 300	70-135	110.2 ± 34.85	125.7	
Creatinine improvement	**Not improved**	70-222	138.25 ± 62.9	130.15	-
	Improved	-	-	-	
Alb/creat ratio improvement	**Not improved**	70-222	138.25 ± 62.9	130.2	-
	Improved	-	-	-	
Osteolytic lesions	**Yes**	222	222	222	0.1
	No	70-194	125.74 ± 40.15	125.7	
Response to MM treatment	**CR**	135-165	149.55 ± 21.14	149.55	0.7
	VGPR	99-155	125.5 ± 39.59	126.5	
	PR	70-183	136.18 ± 43.67	134.6	
	PD	80-103	91.34 ± 15.93	91.34	
	ST	73-194	117.63 ± 52.8	101.65	

Table 4a. Treatment of MM patients in relation to overall survival

Treatment	Number of Patients	OS months		
		range	(mean±SD)	median
VCD	15	1-156	47.2±49.02	37.53
VRD	2	14-38	25.9±16.5	25.8
VTD	2	62-77	69.4±10.6	69.38
Endoxan-Dexa	4	2-19	7.35±7.9	4.07

*: Significant at $P \leq 0.05$

VCD=Velcade® (bortezomib)-Cyclophosphamide-Dexamethasone; VRD=Velcade® (bortezomib)-Revlimid® (lenalidomide)-dexamethasone;VTD=Velcade® (bortezomib)-Thalidomide-dexamethasone;Endoxan-Dexa=Endoxan- dexamethasone

Table 4b. Treatment of MM patients in relation to disease-free survival

Treatment	Number of Patients	DFS months		
		range	(mean ± SD)	median
VCD	13	0-147	35.9±48.6	20
VRD	2	9-21	15±8.5	15
VTD	2	56-67	61.5±7.8	61.5
Endoxan-Dexa	4	0-16	4±8	0

*: Significant at $P \leq 0.05$

VCD=Velcade® (bortezomib)-Cyclophosphamide-Dexamethasone; VRD=Velcade® (bortezomib)-Revlimid® (lenalidomide)-dexamethasone;VTD=Velcade® (bortezomib)- Thalidomide-dexamethasone;Endoxan-Dexa=Endoxan-dexamethasone

Table 5a. Kaplan Maier analysis for OS between the VEGF-A & CXCL13 levels

	Mean OS	95% CI	Median	95%CI	*P*
Low VEGF-A	98.53	46.9, 150.2			0.89
High VEGF-A	44.88	30.3, 59.4	40.6	34.3, 46.9	
Low CXCL13	-	-	-	-	-
High CXCL13	115.5	76.3, 154.8			

*: Significant at $P \leq 0.05$

Table 5b. Kaplan Maier analysis for DFS between the VEGF-A & CXCL13 levels

	Mean DFS	95% CI	Median	95%CI	*P*
Low VEGF-A	74.6	24.4, 124.8	9	2.8, 29.2	0.24
High VEGF-A	18.2	9.4, 26.9	16	0, 33.3	
Low CXCL13	-	-	-	-	-
High CXCL13	54.4	21.9, 86.8	22	4.9, 39.1	

*: Significant at $P \leq 0.05$

Table 6a. Correlations between VEGF-A fold with creatinine, Alb/creat ratio (baseline, after 3 ms, change rates) & β2 microglobulin

	Items	Correlation Coefficient	*P*
VEGF-A	**Creatinine (baseline)**	-0.11	0.48
	Alb/creat ratio (baseline)	0.132	0.49
	Creatinine (3ms)	-0.046	0.81
	Alb/creat ratio (3ms)	-0.39	0.08
	Creatinine change	-0.122	0.52
	Alb/creat ratio change	-0.446	**0.043***
	β_2 microglobulin	-0.123	0.53

*: Significant at $P \leq 0.05$

Table 6b. Correlations between CXCL13 fold with creatinine, Alb/creat ratio (baseline, after 3 ms, change rates) & β2 microglobulin

CXCL13	Items	Correlation Coefficient	*P*
	Creatinine (baseline)	0.238	0.342
	Alb/creat ratio (baseline)	1	-
	Creatinine (3ms)	0.211	0.789
	Alb/creat ratio (3ms)	1	-
	Creatinine change	0	1
	Alb/creat ratio change	-0.4	0.6
	β_2 microglobulin	-0.058	0.837

*: Significant at $P \leq 0.05$

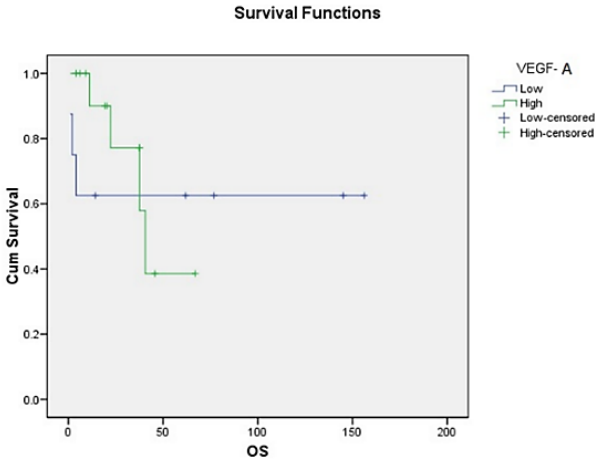

Figure 1. Kaplan Maier analysis for OS regarding VEGF-A level

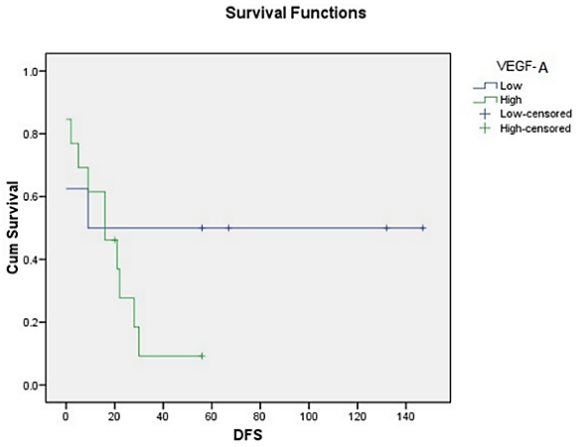

Figure 2. Kaplan Maier analysis for DFS regarding VEGF-A level

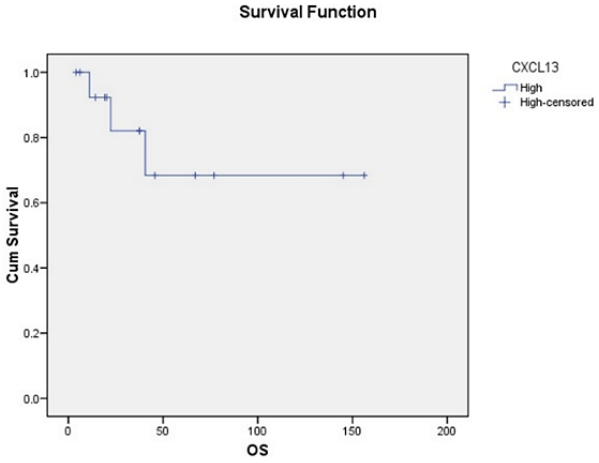

Figure 3. Kaplan Maier analysis for OS regarding CXCL13 level

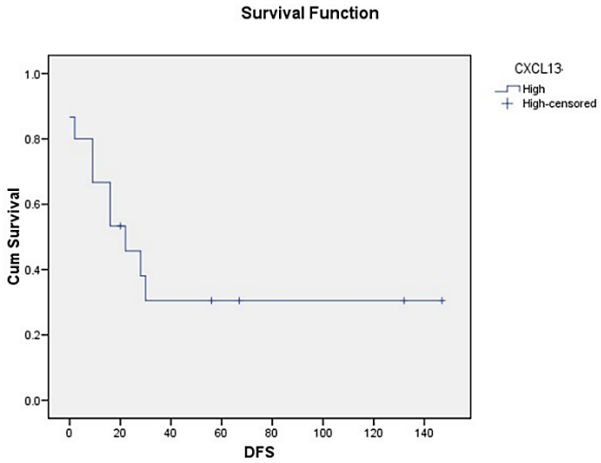

Figure 4. Kaplan Maier analysis for DFS regarding CXCL13 level

Correlation between VEGF-A and CXCL13 MM patients

There was an indirect proportional relation between VEGF-A and CXCL13 levels in myeloma patients with correlation coefficient (r= -0.27). However, it did not reachsignificant statistical correlation (*P*=0.22) as shown in figure 5. Correlations between VEGF-A or CXCL13 folds with creatinine, Alb/creat ratio (baseline, after 3 ms, change rates) and $\beta_2$microglobulin was described in table 6. Only, alb/creat ratio change showed indirect proportional relation with VEGF-A. Statistical analysis revealed significant correlation between VEGF-A and Alb/creat ratio change (*P*=0.043*).

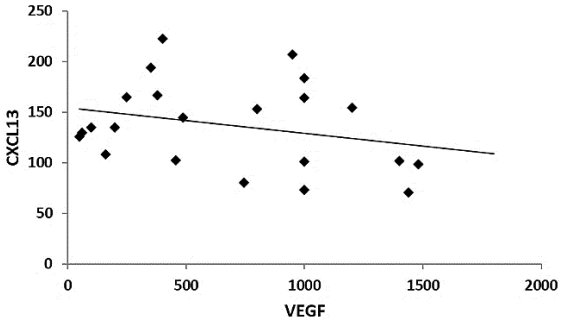

Figure 5. Correlation between VEGF-A and CXCL13 levels

Discussion

Different antiangiogenic agents have changed clinical practice for both the newly diagnosed and the relapsed myeloma patients. However, these agents showed variable degrees of clinical efficacy, but further results confirm the angiogenesis effect in MM pathogenesis (13). Also, myeloma cells express many chemokine receptors and secrete several chemokines that was implicated in cell homing, tumor growth, and progression. Myeloma cells migration to and from BM, as well as their chemotaxis in the BM microenvironment, is controlled through interaction between these chemokine receptors and their ligands (14). Our study aimed at investigating 2 serum biomarkers: VEGF-A and CXCL13 levels in Egyptian myeloma patients as well as correlation with different clinic-pathological features, overall survival, disease-free survival and therapy outcome. In our study, VEGF-A level was significantly higher in MM cases compared to controls (*P*=0.04*). This is in agreement with Shen *et al*., 2005 who revealed that serum VEGF concentrations in MM & solid tumor patients were significantly higher than healthy volunteers (*P*<0.01*), and the VEGF level was higher in myeloma than in solid tumor patients with bone metastasis (15). Our results revealed that patients

who were >65 years old had higher serum VEGF-A levels than younger patients with no statistical significance among the 2 groups (*P*=0.64). This contrasts with Li *et al*., 2014 who reported that patients who were <65 years had higher serum VEGF levels than elder patients, and also, had no statistical significance (*P*>0.05) (16). In our MM patients, VEGF-A levels in Stage II were insignificantly higher than in Stage I and lower than in Stage III. However, Li *et al*., 2014 showed that patients of ISS stage I had lower VEGF level than that of stage II and III with no statistical difference (*P*>0.05) (16). Usnarska-Zubkiewicz *et al*., 2003 reported that patients with stage III had significantly (*P*<0.05) higher VEGF level than those in stage II, also, Shen *et al*., 2005 showed that VEGF levels in Stage II were significantly higher than in Stage I (*P*<0.05) (15,17). The studied group of MM patients with creatinine level ≥2 mg% had insignificantly higher VEGF-A level than those with normal renal function, *P* 0.9. Regarding Alb/creat ratio improvement, median VEGF-A level was significantly higher in patients who improved. This agrees with Usnarska-Zubkiewicz *et al*., 2003 who revealed that MM patients with renal failure (creatinine level >2 mg%) had higher VEGF level than those with normal renal function, however, it reached significant difference, *P*<0.01. It is known that VEGF is a poor prognostic factor for chemotherapy outcome. VEGF plays an essential role in maintaining the blood supply for growing and metastasizing tumors (18). Our findings revealed that after treatment, patients obtained complete remission (CR) had insignificant lower median serum VEGF-A level compared to other patients, *P*=0.2. Also, patients with high VEGF-A levels had short disease-free survival (DFS) and overall survival (OS) time. Li *et al*., 2014 showed that patients who reached complete remission (CR) or very good partial remission (VGPR) had low serum VEGF level, and those with less than partial remission (PR) had high serum VEGF level. Patients with high VEGF levels had short OS time with statistical difference (*P*=0.03) (16). Regarding CXCL13 level, it was significantly higher in MM cases in comparison to controls with *P*=0.01*. Beider *et al*., 2016 reported increased CXCL13 level in plasma of MM patients and Zhang *et al*., 2020 found that CXCL13 level in MSCs from MM patients was significantly higher than that from controls (19,20). Also, Bürkle *et al*., 2007 found significantly higher CXCL13 levels in serum from CLL patients compared to controls (21). In our study, all MM patients had high CXCL13 level with patients who were >65 years had higher serum CXCL13 levels than younger patients with no statistical significance among the 2 groups (*P*=0.18). Also, CXCL13 levels in Stage II were insignificantly higher than in Stage I and III. Our findings revealed that after treatment, patients obtained complete remission (CR) had insignificant higher median serum CXCL13 level compared to other patients, *P*=0.7. An indirectproportional relation between VEGF-A and CXCL13 levels in myeloma patients was found (r= -0.27, *P*=0.22).

In conclusion, production of variety of growth factors and cytokines such as VEGF-A and CXCL13 was higher in MM patients. Analysis of serum VEGF-A and CXCL13 may be helpful in evaluating the disease status of myeloma patients However, our experiment has to be done on larger sample size and extended period of follow up to validate the participation of the VEGF-A and CXCL13 in disease progression and clinical outcome.

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