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ORIGINAL ARTICLE

THE INTERPLAY BETWEEN C-MYCONCOGENE EXPRESSION AND CIRCULATING VASCULAR ENDOTHELIAL GROWTH FACTOR, ITS ANTAGONIST RECEPTOR, FUNS-LIKE TYROSINE KINASE 1 IN DIFFUSE LARGE B CELL LYMPHOMA: RELATIONSHIP TO PATIENT OUTCOME

By

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Aim: *is to assess the relation between c-Myc oncogene expression and angiogenic factors namely vascular endothelial growth factor (VEGF), funs-like tyrosine kinase 1(Flt-1) in patients with diffuse large B cell lymphoma (DLBCL) and their impact on the patient outcome.*

Methods: *Forty Five DLBCL patients beside 10 normal controls were included. c-Myc oncoprotein was assessed by immunohistochemistry and sVEGF, and sFlt-1 were assessed by immunosorbent assay.*

Results: *c-Myc over-expression was detected in 66.6% of DLBCL. The DLBCL patient group with positive c-Myc over-expression showed significantly higher sVEGF and significantly decreased sFlt-1 as compared to group with negative c-Myc over-expression (P=0.000, 0.009 respectively). sVEGF was positively correlated to sLDH and .v./32 microglobulin (r =0.6, p=0.000, r =0.69, P= 0.000) respectively. The non-survived DLBCL group showed significantly higher expression of c-Myc , high concentration of sVEGF and lower concentration in sFlt-1 as compared to the living group (P=0.000 for all).*

Conclusion: *These findings confirm the in vitro based suggestion that c-Myc over-expression orchestrate the angiogenic switch necessary for tumor progression. c-Myc over-expression, elevated sVEGF, and normal sFlt-1 at diagnosis are poor prognostic markers in DLBCL patients.*

Keywords: *Lymphoid neoplasm, Tumor marker, Prognosis*

INTRODUCTION

Diffuse large cell lymphomas (DLCL) are the most frequently occurring types of NHLs. According to WHO classification of neoplastic diseases of the hematopoietic and lymphoid tissues, DLBCL comprises about 40% of adult cases of non-Hodgkin's lymphoma.(1) This type of NHL is a very heterogeneous group of disorders regarding clinical behavior and histopathologic features than other group of lymphomas, representing the classical high-grade lymphomas. They typically present with rapidly progressive lym-phadenopathy associated with fast cellular proliferation and progressive infiltration. Some patients have a more aggressive course than others.(2) Despite extensive effort and constant progress in our understanding of NHL pathogenesis, the DLBCL group re-mains heterogeneous entity awaiting further clinico-pathological stratification. The working formulation has separated DLBCL into different histologic categories. This histologic distinction is often difficult, not reproducible and does not reflect different clinical outcome. The recently proposed WHO classification of lymphoid neoplasms has grouped all these types in a specific category under term DLBCL.(1) The clinical heterogeneity of DLBCL is likely to be the clinical reflection of several intrinsic biological characteristics of tumor.(3) Molecular events in DLBCLs

also are very complicated. Rearrangement of the c-Myc oncogene frequently involving t(8,14) q24, q32, leading to deregulation and overexpression of c-Myc oncogene.

The proto-oncogene c-Myc encodes a transcriptional factor that is implicated in various cellular process- cell growth, proliferation, loss of differentiation and apoptosis(4-6) Deregulated expression of c-Myc family genes occurs in a broad range of human cancers and is often associated with poor prognosis indicating a key role for this oncogene in tumor progression.(7-10)

For tumor growth and metastasis.(11-13) Tumor angiogenesis was posThe tumor angiogenesis and vasculogene-sis are an essential step tulated, to be regulated by balance between pro-and anti-angiogenic growth factors.(14) Recent data obtained from in vitro observation(15) indicate that tumor angiogenesis can be induced by cellular c-Myc oncogene, leading to the enhanced activity of molecules stimulating angiogenesis. However, activated oncogenes might also facilitate angiogenesis by down-regulating endogenous inhibitors of angiogenesis.(16) The main angiogenic factor is VEGF which exerts its biological function through its receptor Flt-1 (funs-like tyrosine kinase 1). The soluble form was stated to be naturally produced antagonist.(17) Kendall et al(18) reported that mRNA for a soluble truncated form of Flt-l(sFlt-l) was generated by alternative splicing in cultured human umbilical vein endothelial cells. To our knowledge the relationship between sVEGF, sFlt-1 concentrations and c-Myc expression in DLBCLs was not previously characterized.

PATIENTS AND METHODS

This study included 45 newly diagnosed DLBCL patients (26 males, 19 females), their ages ranged from 38 to 70 years (median 56) Table 1. beside 10 control subjects with non specific lymphadenopathy matched in age and sex with the patients. Lymph node biopsies were performed in all patients. The initial diagnosis was confirmed by two pathologists. All patients included in this study were subjected to history taking to verify the presence of B symptoms (fatigue, weight loss and weakness). Physical examination to determine dies presence of lymphadenopathy, or hepatosplenomegaly; standard blood tests which include complete blood counts, erythrocyte sedimentation rate, bone marrow aspiration/biopsy, serum LDH. Serum p2 microglobulin, assessment of the of c-Myc oncogene expression, and sVEGF, sFlt-1 concentrations. In addition, radiological investigations were performed which include abdominal ultrasound, computerized tomography (CT), and chest X-ray. The DLBCL patients were followed up to 36 months or until patients' death (median 18 months). Patients were classified according international prognostic index (IPI)(18) [age, clinical stage, performance status, high sLDH, and number of extranodal site]. IPI was scored as low-intermediate low [scores 0,1,2] and high-intermediate high score [scores, 3,4,5]. The DLBCL patients were classified according to clinical state at 36 months follow up into non-survivors and survivors. The state of survived group was as follows: Patients in complete remission (CR.), which was defined as normalization of physical and radiological findings 4 weeks after the last cycle of chemotherapy. Patients were in partial response (PR), which was defined as 50% reduction of the initial tumor mass. Nineteen patients who did not fulfill the above-mentioned criteria were considered non-responders (NR).

Methods: Venous blood samples (8 ml) were collected by venipuncture from the anticubital vein with minimal hemostasis and were divided as follows: 1 ml blood was added to EDTA tube for complete blood count assay (Coulter counter, Onyx); 3 ml were added to heparinized tube to separate platelet-free plasma which was stored at -80 C until sVEGF and sFlt-1 assay; 2ml to sodium citrate tube for erythrocyte sedimentation rate assay (Westergren method); the remaining blood sample was left to clot. The serum was obtained from clotted sample for determination of sLDH (Autoanalyzer, American Corp Indianpolis) and S./J2 microglobulin (EL1SA).

Immunosorbent assay for sVEGF and sFlt-1: Plasma VEGF and Flt-1 were measured by ELISA using commercially available reagents and recombinant reagents (R&D systems, Abingdon, UK) as described in the manufacturer sheet. The VEGF assay has a minimum sensitivity of 10 pg/ml, with an in-tra-assay coefficient of variation (CV) of 4.9%and inter assay CV of 6.4% at 1.6 pg/ml. The sFlt-1 assay has minimal sensitivity of 50 pg/ml, an intra-assay CV of 3.7%, and inter-assay CV of 8.8%at 10 ng/ml.

Immunosorbent assay of B2 microglobulin: Serum B2 microglobulin was determined by a kit obtained from Eurogenetics, Germany according to manufacturer's instructions. Briefly, predilution of the samples into 10 (10 ul serum 100 ul diluent) were done, then 25 ul samples and standards and 100 ul of enzyme conjugate were added into precoated wells and incubated for 30 minutes at room temperature. After 5 washes, 100 ul of buffer solution containing hydrogen peroxide and 100 of tetramethylbenzidine were added, and color was allowed to develop within 10 minutes at room temperature. The reaction was stopped by adding 50 ul of 2N HC1, and the absorbence of each well was determined at 450 nm.

Determination of c-Myc expression by immunohistochemistry

c-Myc was searched for by specific monoclonal antibody IgG2 (clone 9 El 1 from Nov-ocastra laboratories LTD, Newcastle, UK). The detection kit (chromogen) was obtained from lab. Vision, Fremont, CA, USA.

Procedure: For each lymph node biopsy block, 2 um-thick sections were cut on neo-prene-coated slides. The slides were de-paraffinized and blocked for endogenous per-oxidase (which may produce non specific background staining) with hydrogen peroxides for 20 minutes; then washed 2 times in phosphate buffer solution (PBS). Antigen retrieval was performed using Biogenex antigen retrieval citra solution in a 90 C water bath for 30 minutes. The slides were then allowed to cool for 20 minutes, then block by normal horse serum for 5 minutes at 37 C (to block non specific background staining). The monoclonal antibody with 1:100 dilution was applied for 1 hour in humid medium at room temperature, then washed 4 times in PBS, followed by a biotinylated goat antipolyvalent secondary antibody for 15 minutes at 37° C and washed 4 times in PBS, then Strep-tavidin peroxidase was applied for 100 minutes at room temperature, then washed 4 times in buffer. 1-2 drops Diaminobenezidine (DAB) chromogen was added for 20 minutes at room temperature. Slides were counter-stained with hematoxylin, dehydrated and cover slipped. Burkitf s lymphoma specimen was used as positive control.

Interpretation of the results: c-Myc positive staining was seen as fine brownish granules. The expression was scored semiquantitatively by analysis of 5 -10 higher power fields by two independent observers. The results were scored as negative (0): if no staining was observed in any cell, or the positivity was detected in nonneoplastic cells or only in the cytoplasm. In contrast results were scored as positive, if neoplastic cells exhibited nuclear staining .The intensity of c-Myc expression was graded according to Pagnano et al[19] into (+) if 1- 9 % of the cells were stained;(++) if 10-50% of the cells were stained; (+++) if more than 50% of the cells were stained.

Statistical analysis: Data were analyzed using SPSS (statistical for social sciences) version 9. Qualitative data were presented as numbers and percent. Fisher's exact test was used to test significant difference between groups. Quantitative data were presented as means ± CD. Mann-Whitney test and Student's t-test w used for comparison between means. Correlation between data was done by Spearman's rank correlation. P<0.05 was considered to be statistically significant.

RESULTS

sVEGF, and sFlt-1, f>2 microglobulin, and sLDH concentration levels

sVEGF ,sFlt-l,p2 microglobulin, sLDH concentrations were significantly elevated in DLBCL patients as compared to normal controls (P= 0.000, 0.007, 0.001, 0.000 respectively Table 2.

Interrelation between c-Myc over expression, sVEGF, and sFlt-1 levels

sVEGF was significantly higher among DLBCL patients with positive c-Myc over-expression as compared to those with negative c-Myc over expression (P=0.000). In contrast sFlt-1 was significantly higher in c-Myc negative DLBCL group as compared to DLBCL patients with positive c-Myc over expression(P=0.009) Table 3.

When sVEGF and sFlt-1 were classified into normal and high levels (more than mean normal control+2SD), all the patients with c-Myc overexpression have high VEGF. While, in negative c-Myc over expression group; 5 patients have high sVEGF and 10 have normal sVEGF. Moreover all c-Myc positive patients have normal sFlt-1. On the other hand, among patients group with c-Myc negative over expression, 14 patients have normal sFlt-1 and 16 have high sFlt-1. This means

That c-Myc overexpression always associated with increase in sVEGF, and absence of c-Myc over expression always associated with increased sFlt-1 Table 4.

Relation of c-Myc over expression, sVEGF, and sFIt-1 to severity markers in DLBCL

sVEGF was correlated positively with severity markers in DLBCL being higher in higher age group (60 years) as compared to lower age group (P= 0.000); in DLBCL with bulky disease as compared to non bulky disease (P= 0.000), in stage (3+4) versus stage(l+2) (P= 0.000); in high-high intermediate IPI score, versus low-low intermediate score (P= 0.000), but sVEGF did not differ between DLBCL with positive B symptoms as compared to those with negative B symptoms (P > 0.05). In contrast, Flt-1 was correlated negatively with severity markers in DLBCL being lower in higher age group (60 years) as compared to lower age group (P= 0.000); in DLBC1 with bulky disease as compared to non bulky disease (P= 0.000), in stage (3+4) versus stage (1-2) (P= 0.007); in high-high intermediate IPI score, versus low- intermediate low score (P =0.013), but sFlt-1 did not differ between DLBCL with positive B symptoms as compared to those with negative B symptoms (P> 0.05) Table 5.

c-Myc over expression was significantly higher in DLBCL patients aged 60 as compared to those <60 years (P =0.00) , in bulky disease versus non-bulky disease (P=0.002), in stage (3+4) versus(1+2) (P=0.00), in high-high intermediate IPI score as compared to low-low intermediate IPI score (P=0.01). However, C-Myc over expression was not significantly different in DLBCL patients with positive B symptoms as compared to those with negative B symptoms (p>0.05, Table 6.

VEGF was positively correlated with sLDH and β2 microglobulin (r =0.6, P=0.000, r =0.69, P= 0.000 respectively); but negatively correlated to sFlt-1 (r - 0.58, P= 0.000). On the other hand, sFlt-1 was negatively correlated to sLDH, and sβ2 microglobulin r - 0.25, P >0.05, r - 0.48, P= 0.001) Table 7.

Relation of c-Myc, sVEGF, sFlt-1 to patients outcome: sVEGF and c-Myc over expression were significantly higher in non-survived DLBCL as compared to living patients (P= 0.000 for both). (Fig. 1). In contrast, Flt-1 was significantly higher in living DLBCL (P=0.0000) as compared to non-survived group. (Fig. 2). c-Myc over expression and elevated s.VEGF were present in all died patients Moreover, in the non-survived group 12 out of 19 have normal sFlt-1.

Table 1. Some relevant data of studied patients (n= 45).

	No	%
Age		
≥ 60	19	42.2
> 60	26	57.8
sex		
♂	26	57.8
♀	19	42.2
-B-symptoms		
positive	25	55.5
negative	20	44.5
-Bulky disease *		
positive	24	53.3
negative	21	46.7
-An Arbor stage		
I / II	18	40.0
II/IV	27	60.0
-IP I score		
Low / low intermediate risk	28	62.2
High / high intermediate risk	17	37.8
Patient status		
Non-survivors	19	42.2
Partial response	8	17.8
Remission	18	40.0

* Bulky disease (> 5cm in diameter).

Table 2. s.VEGE, s.Flt-1, s.p2 microglobulin and s.LDH in DLBCL versus normal controls.

Group	s.VEGF (pg/ml) X± SD	s.Flt-1 (ng/ml) X ± SD	s.p2 microglobulin (ng/ml) X ± SD	s.LDH (u/L) X ± SD
Control (n=10)	133.0± 18.5	36.4 ± 1.8	2.0 ± 0.18	233.8 ± 14.2
DLBCL (n=45)	671.6 ± 53.4	90.6 ± 9.1	4.7 ± 2.3	524.1 ± 23.1
Mann.Whitney test	P= 0.000	P= 0.007	P= 0.001	P= 0.000

Table 3. s.VEGF and sFlt-1 concentration levels among DLBCL patients positive for c-Myc versus those negative for c-Myc over expression.

Group	sFlt-1	sVEGF
c-Myc over expression	X + SD	X + SD
positive	74.1 ±10.9	875.0 ±45.7
negative	123.6 ±12.9	264.7 ±22.3
Mann.Whitney test	P = 0.019	P = 0.000

Table 4. Interrelation between c-Myc over expression & s.VEGF and s.Flt-1 cncentration levels in DLBCL patients.

Parameters	C-Myc over expression				Fisher's exact test
	Negative (n= 15)	%	Positive (n= 30)%		
sVEGF					
Normal	10	66.7	-	-	P=0.000
High	5	33.3	30	100	
sFlt-1					
Normal	-	-100	14	46.7	P=0.000
High	15		16	53.3	

C-myc, sVEGF and s Flt-1 in DLBCL

Table 5. Relation between s .VEGF and s.Flt-1 concentrations and severity markers in DLBCL patients.

	s.VEGF	s.Flt-1
Age		
> 60	967.9 ±64.9	52.1 ±8.5
> 60	455.0 ±45.0	118.8 ±11.8
Mann. Whitney test	p = 0.000	p = 0.000
B-symptoms		
positive	720.2 ±73.2	87.4 ±12.5
negative	610.8 ±77.7	94.7 ±13.5
Mann. Whitney test	P=0.06	P=0.06
Bulky disease		
positive	845.2 ±68.7	66.1 ±9.8
negative	473.1 ±59.5	118.7 ±13.8
Mann. Whitney test	P = 0.000	P = 0.000
IP I score		
Low / low intermediate risk	541.6 ±58.7	107.9 ±11.5
High / high intermediate risk	885.6 ±81.0	62.2 ±12.2
Mann. Whitney test	P = 0.001	P = 0.013
Disease stage		
(1+2)	397.6 ±49.0	119.9 ±12.0
(3+4)	854.3 ±61.5	71.2 ±11.6
Mann. Whitney test	P = 0.000	P = 0.007

Table 6. Relation of c-Myc over-expression to severity markers in DLBCL patients.

	C-Myc over expression				Fisher's exact test
	Negative (15) N	%	Positive (30)		
Age					
> 60	14	93.3	12	40	
> 60	1	6.7	18	60	P= 0.001
B-symptoms					
Positive	8	53.3	12	40	
Negative	7	46.7	18	60	P= 0.396
Bulky disease					
Negative	12	80	9	30	
Positive	3	20	21	70	P= 0.002
IP I score					
Low	13	86.7	15	50	
High	2	13.3	15	50	P= 0.01
DLBCL stage					
1+2	11	73.3	7	23.3	
3+4	4	26.7	23	76.7	P= 0.001
Patient outcome					
Non survivors	-	-	19	63.3	
Survivors	15	100	11	36.7	P= 0.000

Table 7. Correlation between concentration levels of s.VEGF, s.Flt-1, s.LDH and B2 microglobulin.

	s.Flt-1	s.LDH	s.B2 microglobulin
	r-0.58	r 0.6	r 0.7
s.VEGF{pgml)	p 0.000	p 0.000	p 0.000
		r-0.25	r 0.62
s.Flt-1 (ng/ml)	----	p=0.385	p 0.00

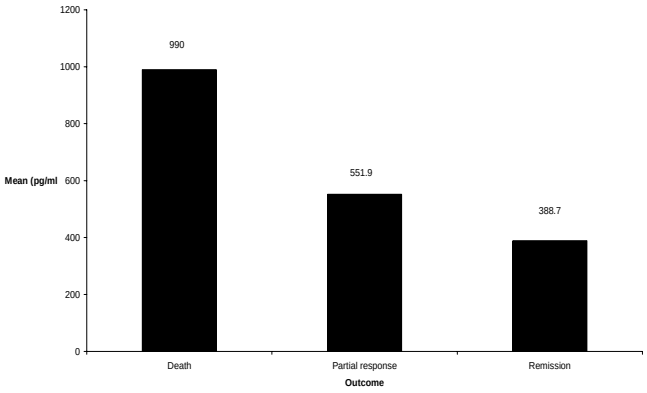

Fig 1. Levels of s.VEGF according to patient outcome.

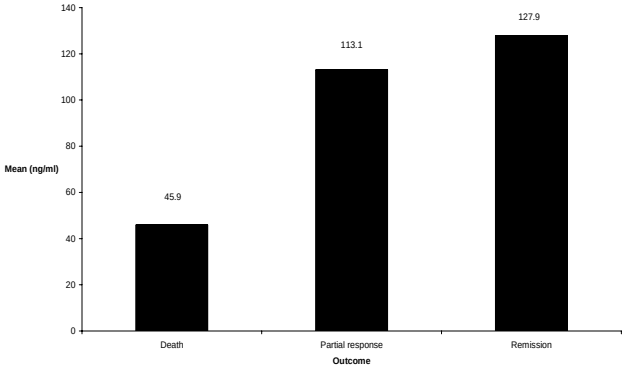

Fig 2. Levels of s.Flt according to patient outcome.

DISCUSSION

c-Myc is a multifunctional proto-oncoge-ne, and its constitutive expression results in oncogenic activation and contributes to progression of a wide range of human and animal tumors. However, the known c-Myc functions which are cell cycle control and regulation of apoptosis was claimed to be insufficient to promote tumorigenesis, and that c-Myc oncoprotein must provide other functions that initiate and or/sustain malignancy. Recently, Baudino et al t,[15] in an experimental study reported a novel angiogenic function for c-Myc .To assess this function in clinical sample we have planned this study.

The c-Myc oncogene over expression was observed in 66.6 % of the studied DLBCL patients group. Similar finding was reported by Pagnano et al.[19] On the other hand Kawaski et a l[20]reported lower c-Myc oncogene expression. This difference could be attributed to the difference in the method of detection. The role of c-Myc oncoprotein in lymphomagenesis is complex. In addition to established function of c-Myc over expression that leads to cell proliferation by promoting the expression of a set of cell cycle activators (cyclin Dl, D2, E, and cdK4), and transcriptional repression of cell cycle/ growth arrest genes (gasl, pl5, p21, p27 and gadd34, -45, and-153)[5] and abolishment of differentiation and apoptosis, recently angiogenic function have been stated.[15]

B2 microglobulin and sLDH were significantly higher in DLBCL group as compared to controls. This finding is in agreement with that of Alici et al. sLDH and $2 microglobulin were reported to be indirect indicators of tumor mass as well as markers of disease activity.[22,23] Angiogene-sis, the formation of new blood vessels, is controlled by balance between positive and negative endothelial regulatory factors.

Soluble VEGF and sFlt-1 were significantly elevated in DLBCL patients as compared to controls. This finding is in accordance partially with the results of Bel-gore et al[24] that measured by ELISA sVEGF and sFlt-1 in hematological cancer and breast cancer and concluded that sVEGF but not sFlt-1 levels were significantly higher in both diseases as compared to controls. Belgore et al[24] also stated that the levels of VEGF were higher in hematological cancer as compared to breast cancer. Moreover, Barleon et al[25] reported the presence of sFlt-1 in normal human serum and suggests that sFlt-1 plays an important role as a naturally occurring VEGF antagonist in the regulation and availability of VEGF-mediated biological activities in vivo.

In the present study VEGF was significantly higher in c-Myc positive DLBCL patients as compared to c-Myc negative patients. Moreover, all patients with c-Myc over expression are associated with elevated sVEGF. On contrary Flt-1 was significantly lowered in c-Myc positive patients as compared to those patients with negative c-Myc over expression. In addition, all patients with normal or low sFlt-1 were positive for c-Myc over expression. This means that c-Myc over expression associated with elevation of angiogenic factors and suppress the angiogenic inhibitors. These findings confirm the in vitro reported findings by Bau-dino et al.[15] Furthermore, Toi et alc[26] assessed the expression of VEGF and VEGFR1 (Flt-1) in breast cancer, they found high expression of VEGF and VEGFR and concluded that the intratumoral balance between sVEGFRl and VEGF levels might be crucial for the progression of breast cancer.

The precise mechanism by which c-Myc controls the expression of angiogenic factors is not resolved, yet VEGF regulation appears to be indirect. It was reported that c-Myc indirectly regulates VEGF RNA levels by affecting the turnover of VEGF RNA. Regulation of VEGF RNA stability during hypoxia and in other scenarios is well documented.[27-28] However, Baudino et al[15] suggested that VEGF gene might be a transcriptional target for c-Myc oncoprotein, and they reported that c-Myc over expression augment VEGF expression. Moreover, c-Myc augments angiogenesis through disabling the expression of Flk-1, and Flt-1.

The increased soluble sFlt-1 in c-Myc DLBCL negative patients may have biological function as to regulate plasma VEGF levels (18'29'30). The inhibitors function of sFlt-1 to VEGF has been emerged in several studies. Goldman et al[30] reported that native soluble VEGFR inhibits tumor growth, metastasis, and mortality rate. In addition Hasumi et al[31] stated that soluble Flt-1 expression suppresses carcinomatous ascites in nude mice bearing ovarian cancer and suggested that inhibition of VEGF activity by sFlt-1 expression may provide a means to control carcinomatous ascites and angiogenesis of malignant ascites tumors.

All patients with increased VEGF care present among c-Myc positive group. On the other hand all c-Myc negative patients have high sFlt-1. This supports the in vitro suggestion that C-Myc oncogene regulates an-giogenesis through inducing expression of VEGF and suppression of sFLt-1. However among the c-Myc positive patients there are 16 patients have high sFlt-1; this may point for another mechanism controlling sFlt-1 expression or the degree of expression of c-Myc is not sufficient to suppress sFlt-1.

Baudino et al[15] stated that c-Myc loss impairs vasculogenesis and angiogenesis and reduction in VEGF expression. They suggest that VEGF might be a transcriptional target of c-Myc and they found that that overexpression of c-Myc also augments VEGF expression. Not only this but also, they stated that c-Myc is required for proper expression of the angiogenic network. They proved also that c-Myc disabled the expression of cellular F1K-1 and Flt-1.

c-Myc over expression, and sVEGF were significantly higher among non -survived group as compared to living group. In contrast living group has significantly higher sFlt-1 as compared to non-survived group. These findings are consistent with previous studies.[32,35]

In conclusion, we found an association between c-Myc overexpression, increased VEGF, normal sFlt-1 and disease severity markers. This confirms the in vitro suggestion that c-Myc over expression orchestrates the angiogenic regulators through increasing sVEGF and suppressing sFlt-1 production. c-Myc over expression, high serum VEGF and normal s.Flt-1 at diagnosis are associated with poor outcome in DLBCL.

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