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Prognostic relevance of CD69, Vascular endothelial growth factor and Thymidine kinase in B-chronic lymphocytic leukemia

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ABSTRACT

Background: B-chronic-lymphocytic-leukemia (B-CLL) is a heterogeneous illness with a varied clinical history. Some individuals don't require therapy while others have an aggressive disease. Researchers discovered several prognostic biomarkers to help clinicians make decisions about whose patients will be in need to start treatment. Developing immunophenotyping markers such as CD69 and other serum markers such as TK and VEGF are evolving over time.

Objective: This study aimed to assess relevance of CD69 as prognostic indicator in CLL cases and its association with Vascular-endothelial-growth factor (VEGF) and Thymidine-kinase (TK) activity.

Methods: The research had 80 patients with B-CLL and 17 healthy controls. All subjects underwent routine laboratory investigations, flowcytometric analysis of CLL panel, CD38 and CD69 and TK activity and VEGF level by ELISA**.**

Results: CD69 %, VEGF and TK were significantly higher in patients than in controls. Patients who required treatment had significantly higher CD69% and elevated levels of VEGF and TK than patients who were untreated. Correlation between CD69% with different parameters revealed significant positive correlation between CD69% and age, modified Rai staging, TLC, lymphocyte, B2 microglobulin, LDH, Coombs`test, receiving chemotherapy, CD 38/19%, VEGF and TK. CD69% at cut off levels of > 45% can significantly detect time to start chemotherapy with sensitivity and specificity of 88.89% and 77.27%. Regression analysis showed that age, modified Rai staging, lymphocyte count and HB level were independent variables that can predict CD69 expression. **Conclusions:** CD69, TK and VEGF could be considered as poor prognostic markers adding a new option for development of new prognosis score system.

Keywords: Chronic lymphocytic leukemia, Biomarkers, CD69, Prognosis, Scoring.

INTRODUCTION

B-chronic lymphocytic leukemia (CLL) is defined by an excess of monoclonal B lymphocytes with the CD19+/CD5+/CD23+ phenotype in lymphoid tissue, peripheral blood, and bone marrow. According to statistics, CLL accounted for 25–30% of leukemia diagnoses, giving it the most common kind of leukemia in adults in North America and Europe [(1)]. The illness is diverse and has a varying clinical history; some people have an indolent course and don't need treatment, while others have an aggressive illness with a short duration and overall survival [(2)]. As a result, researchers discovered several prognostic biomarkers and staging systems to help clinicians make decisions about whose patients will be in real need to start treatment. These biomarkers include serum markers, immunophenotyping markers, microRNAs, chromosome aberrations, IGHV mutation status and gene mutations [(3)]. ZAP-70 and CD38 are established immunophenotyping markers that can predict aggressive stages. Conversely, leukemia tends to develop slower and have better prognosis when the CLL cells are deficient in these proteins [(4)]. Other developing immunophenotyping markers such as CD69 are evolving over time. CD69 is a human transmembrane C-type lectin protein produced by the CD69 gene located in the natural killer (*NK*) gene cluster at chromosome 12. It is expressed as an activation marker in hematopoietic stem cells [(5)]. Given that CD69's production is elevated following activation in the majority of leucocytes and it regulates immunological responses, it is employed as an activity indicator of active lymphocytes and NK cells. [(6)]. VEGF is an important positive mediator of physiological and pathological angiogenesis. It has a tight connection to human tumorigenesis and the development of many malignancies. Solid tumor development, diffusion, metastasis, and a poor prognosis are connected to elevated VEGF levels in blood. In hematological malignancies like multiple myeloma, CLL, non-Hodgkin lymphoma and chronic myeloid leukemia, its levels are increased [(7)]. It activates mitotic responses as it stimulates growth, migration, and survival and it improves hematologic malignancies' leukemia progenitor cells' capacity for self-renewal [(8)]. One of the tumor indicators that may be detected in constantly proliferating cells is thymidine kinase (TK). Serum TK activity is elevated in many types of cancer. Also, TK was utilized in CLL as a diagnostic indicator [(9)].

Various research showed the importance of CD69 and CD38 expression as prognostic indicators in CLL. Enhanced serum TK and VEGF level was found also in patients with advanced disease stage [(10)].So, we conducted this research to evaluate relevance of CD69 as prognostic indicator in B-CLL cases and its association with VEGF, TK activity.

SUBJECTS AND METHODS

This case control research was conducted in the Clinical Pathology Department, Faculty of Medicine, Menoufia University between November 2020 and October 2021. The patients were selected from Inpatient Wards and Outpatient Clinics, Oncology Unit, Menoufia University Hospitals. The study included 97 subjects divided into two groups: **Group I:** included 80 CLL patients presented to Oncology Department and **Group II:** included 17 healthy subjects of matched age and sex as control group. **All patients were subjected to the**

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following: Complete history, clinical evaluation, routine laboratory investigations [Complete blood picture measured by hematology autoanalyzer Sysmex XN-1000 (Sysmex Corporation, Japan's Kobe) and blood film, lactate dehydrogenase (LDH) assay done by autoanalyzer AU 680 (Beckman Coulter, AU Chemical Analyzer, USA), B2 microglobulin (B2M) assay done using one step immunoassay on Minividas (biomeriieux, Paris , France) and Immunophenotypic analysis of patients samples on cytoFLEX flowcytometer (Beckman Coulter Inc, USA) using chronic lymphoproliferative panel which includes CLL scoring system (CD5/CD19, CD23, FMC7, CD79b, CD22, surface immunoglobin, CD20**,** CD10, CD103, CD123, Kappa/Lambda light chains) and CD69 and CD38. VEGF and Thymidine kinase detection were assessed by enzyme linked immunosorbent assay (ELISA). Diagnosis of CLL was established by persistent lymphocytosis for at least $5x10^9$/L in the peripheral blood with blood film showed characteristically small, mature looking lymphocytes and smudge cells. Immunophenotyping was furtherly investigated to confirm diagnosis of CLL and to distinguish it from other chronic lymphoproliferative disorder following CLL scoring system (CD5, CD23, FMC7, CD79b and surface immunoglobin). For laboratory investigations and immunophenotyping: 5 ml of peripheral venous blood was drawn under complete aseptic condition form all the subjects and divided into 2 tubes: 2 ml on ethylenediaminetetraacetic acid (EDTA) tubes for CBC, blood film and flowcytometric analysis, the other 3 ml on plain vacutainer allowed to clot and separated serum was used for LDH, B2 microglobulin assays and the remaining serum was stored in -25 ˚C for further assessment of VEGF and Thymidine kinase 1 by ELISA. **For flowcytometric analysis:** blood samples were resuspended in PBS to a final adjusted white blood cell count of 4-6 $\times 10^3$/uL. Next, 100 µL of diluted samples were vortexed for 3 seconds then incubated with 10 µL of each of the monoclonal antibodies for 20 min at room temperature in the dark. Thirteen monoclonal antibodies were utilized as chronic lymphoproliferative panel: separated into 5 tubes: tube 1 contained fluorescein isothiocyanate (FITC)-conjugated anti-CD5; phycoerythrin (PE)-conjugated anti-CD19; and allophycocyanin (APC)-conjugated anti-CD23. Tube 2: (FITC)-conjugated anti-FMC7; (PE)-conjugated anti-CD79b; and (APC)-conjugated anti-CD22. Tube 3: (FITC)-conjugated anti- CD20; (PE)-conjugated anti-CD CD10; and (APC)-conjugated anti-SIgm, Tube 4: (FITC)-conjugated anti- CD103; (PE)-conjugated anti-CD123; and (APC)-conjugated anti-Kappa/Lambda light chains and tube 5: FITC-conjugated anti- CD69; (PE)-conjugated anti- CD38. A lysis solution was added, vortexed for 10 minutes right away, and then let to sit at room temperature in the dark for another 10 minutes. Cells were fixed by adding 500 µL of 1% PBS-formaldehyde after being washed with PBS. Utilizing a cytoFLEX flowcytometer, immunophenotyping study was carried out (Beckman Coulter Inc, USA). Instrument software was used to do data collecting and analysis. 10,000 or more gated events were collected. To diagnose CLL patients, the modified Matutes score was computed, and positivity was determined to be ≥30% positive cells population. When the number of positive cells was less than 30%, CD5 and CD23 were counted as score 1, whereas FMC7 and CD79b were also given that designation. Additionally, when the expression was poor, sIgM was given a score of 1. A score of < 4 indicated atypical cases, whereas a score of ≥ 4 indicated typical CLL patients [(11)]. For VEGF and Thymidine kinase analysis: VEGF and Thymidine kinase 1 ELISA kits were used (BioVision, Milpitas, USA), following the manufacturer's specifications. The method employed was a quantitative sandwich enzyme immunoassay. An anti-VEGF and anti-TK monoclonal antibodies had been precoated onto a microplate. Standard and samples were pipetted into the wells, where the immobilized antibody binds to any VEGF or TK present. An enzyme associated monoclonal antibody specific for VEGF and TK was introduced to the wells after any unbound compounds have been removed by washing. A substrate solution is added to the wells after a wash to eliminate any unbound antibody enzyme reagent, and color develops according to the quantity of VEGF and TK bound in the first phase. The intensity of color signal is directly proportional to their concentration in the specimen.

Ethical Approval: The study was approved by the Ethics Board of Menoufia University and the patients were given all the information they need about the trial. An informed written consent was taken from each participant in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis: With the aid of the IBM SPSS software package version 20.0, data were fed into the computer and evaluated. (IBM Corp, Armonk, NY). Qualitative data were described using number and percent and were compared using Chi square test or Fisher Exact test. Normally quantitative data was expressed in mean ± SD and were compared using student t-test. Abnormally distributed data was expressed in median (Min. – Max.) and was compared using Mann Whitney test. Statistically significant at $p \leq 0.05$.

RESULTS

A comparison of the two analyzed groups' demographic information is shown in Table (1). Regarding CBC comparison between the two study groups, the control groups had greater hemoglobin levels and platelet counts.

On the other hand, the case group had higher values of WBCs and lymphocytes. Regarding B2 microglobulin and LDH level, CLL group had significantly higher values of B2 microglobulin and LDH compared to control group. Concerning immunophenotyping, a comparison of the two study groups showed that the case group had significantly high CD69%, CD38%, CD69/19%, CD38/19%, and CD38/69% values. Comparison between the two study groups as per VEGF and TK assay level revealed that the case group had

significantly higher values of VEGF and TK than the control (Table 1).

Table (1): Comparison between the two studied groups according to different parameters

		Cases (n=80)	Control (n=17)	P
Sex:	Male	42 (52.5%)	10 (58.8%)	**0.635**
	Female	38 (47.5%)	7 (41.2%)	
Age (years)		63.7 ± 10.7	61.2 ± 11.6	**0.385**
HB (g/dL)		11.4 ± 2.3	13.6 ± 1.1	**<0.001***
TLC (×10^3/uL)		65 (12 – 190)	7.4 (5.1 – 10.3)	**<0.001***
Lymphocytes (×10^3/uL)		56 (8 – 134)	1.9 (1.1 – 2.8)	**<0.001***
Platelets (×10^9/L)		157.2 ± 68.2	262.4 ± 77.5	**<0.001***
B2M (mg/L)		5.0 ± 1.6	1.7 ± 0.55	**<0.001***
LDH (IU/L)		593.5 (190 – 786)	145 (46 – 210)	**<0.001***
CD69 (%)		52.5 (2 – 89)	2.3(0.4 – 4)	**<0.001***
CD69/19 (%)		42.5 (1 – 84)	0.10 (0 – 0.6)	**<0.001***
CD38 (%)		45.5 (2 – 93)	1.5 (0 – 5.2)	**<0.001***
CD38/19 (%)		40 (1 – 90)	0.2 (0 – 2.1)	**<0.001***
CD38/69 (%)		26 (0 – 67)	0 (0 – 0.5)	**<0.001***
Comb's test:	Negative	50 (62.5%)	17 (100.0%)	**0.002***
	Positive	30 (37.5%)	0 (0.0%)	
VEGF (pg/mL)		864 (456 – 1693)	197 (79 – 352)	**<0.001***
TK (ng/mL)		7.6 (2.3 – 12)	1.8 (0.7 – 3.6)	**<0.001***

B2M: B2 microglobulin, VEGF: vascular endothelial growth factor, TK: thymidine kinase *: Statistically significant at $p \leq 0.05$. Mean ± SD for parametric data-media, range for non-parametric data.

Distribution of the study cases as per modified Rai staging revealed that 25% of patients were modified Rai stage 1, 32.5% of patients were modified Rai stage 2 and 42.5% of patients were modified Rai stage 3. Distribution of the study cases as per lymphocyte doubling time (LDT) revealed that 47.5% of patients had LDT ≤ 12 months and 52.5% of patients had LDT >12 months. The distribution of the study cases as per received chemotherapy showed that 45% of patients received chemotherapy and the remaining were untreated.

Correlation study between CD69% and CD38% with different parameters in the case group revealed that there was positive correlation between CD69% and CD38% with age, modified Rai staging, TLC, lymphocyte, B2 microglobulin, LDH, Coombs`test, receiving chemotherapy, CD 69/19%, CD 38/19%, CD 38/69%, VEGF and TK, while significant negative correlation was observed between CD69% and CD38% with platelet count, LDT and HB level (Table 2).

Table (2): Correlation between different parameters in the case group (n=80)

	CD69 (%)		Cd38 (%)	
	r_s	P	r_s	P
Age (years)	0.756	<0.001*	0.656	<0.001*
Modified Rai staging	0.891	<0.001*	0.749	<0.001*
Hb (g/dL)	-0.751	<0.001*	-0.524	<0.001*
TLC (×10^3/uL)	0.619	<0.001*	0.459	<0.001*
Lymphocytes (×10^3/uL)	0.620	<0.001*	0.463	<0.001*
LDT (>12 months)	-0.840	<0.001*	-0.785	<0.001*
Platelets (×10^9/L)	-0.818	<0.001*	-0.770	<0.001*
B2M (mg/L)	0.751	<0.001*	0.614	<0.001*
LDH (IU/L)	0.718	<0.001*	0.656	<0.001*
CD69/19	0.960	<0.001*	0.819	<0.001*
CD38/19	0.835	<0.001*	0.996	<0.001*
CD38/69	0.898	<0.001*	0.947	<0.001*
Comb's test	0.743	<0.001*	0.737	<0.001*
VEGF (pg/mL)	0.858	<0.001*	0.766	<0.001*
TK (ng/mL)	0.790	<0.001*	0.700	<0.001*
Received chemotherapy	0.642	<0.001*	0.575	<0.001*

r_s: Spearman coefficient, *: Statistically significant at $p \leq 0.05$, LDT: lymphocyte doubling time, B2M: B2 microglobulin, VEGF: vascular endothelial growth factor, TK: thymidine kinase.

Stepwise Linear regression analysis showed that age, modified Rai staging, lymphocyte count and HB level were independent variables that can predict CD38% and CD69% expression. Regarding the relation between CD38% and CD69% and different parameters in the case group, they were significantly higher in patients with LDT ≤ 12 months, patients with positive Coombs's test and patients who received chemotherapy. Studying the relation between chemotherapy and CD38 and CD69 in cases group revealed that patients who received chemotherapy had significantly higher percentage of CD38% and CD69% and significantly elevated levels of VEGF and TK than patients who were untreated (Table 3).

Table (3): Relation between CD69, CD38 with different parameters in cases group (n = 80)

	N	Cd69%	p	CD38%	P
Sex					
Male	**42**	66 (2 – 89)	**0.177**	55 (6 – 93)	**0.005***
Female	**38**	41 (7 – 89)		18 (2 – 76)	
LDT					
≤12 months	**38**	78 (53 - 89)	**<0.001***	71 (40 - 93)	**<0.001***
>12 months	**42**	19 (2 - 78)		9 (2 - 76)	
Comb's test					
Negative	**50**	27 (2 – 78)	**<0.001***	12 (2 – 74)	**<0.001***
Positive	**30**	82 (52 – 89)		75 (51 – 93)	
Received chemotherapy					
No	**44**	24 (2 – 83)	**<0.001***	12.5 (2 – 76)	**<0.001***
Yes	**36**	76.5 (17 – 89)		69 (4 – 93)	

Abnormally distributed data was expressed in median (Min. – Max.) and was compared using Mann Whitney test.
*: Statistically significant at p ≤ 0.05, LDT: lymphocyte doubling time.

ROC curves were used to identify a cutoff value of CD38, CD69 to best predict chemotherapy cases. They showed that CD69% and CD38% at cut off levels of > 45% and > 40% respectively can significantly detect the need to start chemotherapy with sensitivity and specificity for CD69 of 88.89% and 77.27% and for CD38 of 88.89% and 81.82%, respectively. Furthermore, VEGF and TK at cut off levels of > 864 pg/mL and > 8.7 ng/mL respectively can significantly detect the need to start chemotherapy with sensitivity and specificity for VEGF of 77.78 % and 77.27 % and for TK of 61.11% and 86.36 % respectively (Figure 1).

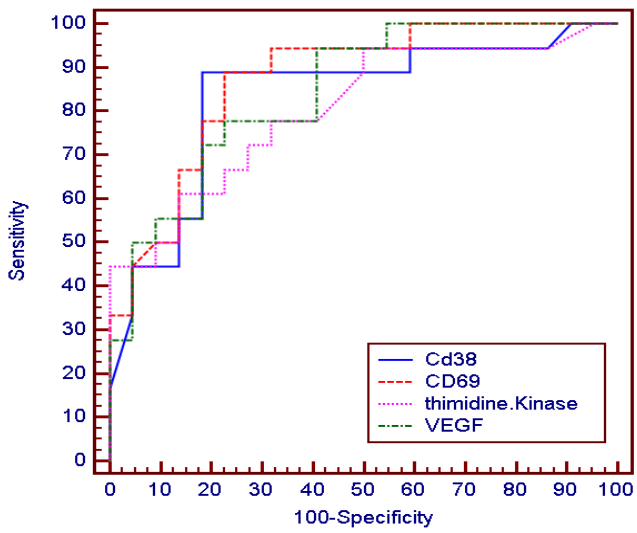

Figure (1): ROC curve for CD69% and CD 38% to predict chemotherapy cases.

DISCUSSION

This study's objective was to evaluate the relevance of CD69 as a prognostic immunophenotyping marker in CLL cases and its association with VEGF and TK activity. There was no statistical variation between cases and control as regards age and sex, which confirms our inclusion criteria of the control group that included healthy subjects with their age and sex matched to the patients group. The mean of our patients` age was 63.70 ± 10.74 years. This comes in line with **Kalpadakis *et al.*** [12] who stated that CLL develops in or after middle age. In the present study male represented 52.5% of the patients` group while female represented 47.5%. This is in accordance with the findings of **Hendy *et al.*** [13]. The higher prevalence of hematological malignancies among males could be attributed to fact that males are more exposed to the occupational agents that increase the risk of hematological cancers development [14]. Serum B2M and LDH levels were significantly higher in patients' group than controls as they directly reflect tumor burden in lymphoma and other cancers. Furthermore, since serum B2M is implicated in the cell survival, growth, and metastasis of cancerous lymphomas, it is assumed to be connected to certain biologic or tumour microenvironmental properties [15]. According to modified Rai staging system, we found that more than 50% of our patients were in stages I and II. This comes

in agreement with **Rai and Keating,** [16] who stated, based on different studies, that about 50% of CLL patients are in stage I or II at diagnosis. Patients` group had significant lower platelets count, and hemoglobin level than controls. The pathophysiology of development of cytopenia in CLL cases relies on many factors including enlarged spleen, autoimmune etiology and/or marrow suppression due to extensive disease infiltration or secondary to drug or chemotherapy [17]. Positive Direct Coombs' test was seen in 37.5% of our CLL patients. This comes in agreement with **Ahmed *et al.*** [18] who stated in his study that DAT might be positive in up to 35% of cases. Performing Direct antiglobin test not only can confirm the autoimmune pathology but also have a role in predicting patients' prognosis as suggested by many authors.

Lymphocyte doubling time (LDT), the time it /takes for the lymphocyte count to double from the first recorded value, has been known to have a diagnostic role in CLL for more than 30 years. The most significant independent predictor for time to first therapy was found to be LDT and have a valuable prognostic significance despite the era of new markers and introducing it to different scoring systems with other molecular and cytogenetic markers, signify their role in predicting patients prognosis and disease follow up [19]. In this study we found 47.5% of patients had LDT < 12 months and 52.5% of patients had LDT >12 month. These percentages are different from what documented by **Baumann *et al.*** [20] in their study aimed to evaluate LDT in 848 CLL patients with regard to its clinical and biological relevance. Among their CLL patients, the percentage of patients with an LDT ≤ 12 months was 11%, while that of patients with a LDT > 12 months was 89%.

CD69 is a type II integral membrane protein with a single transmembrane domain that serves as an immunoregulatory molecule expressed on most hematopoietic cells [21]. In this study, the patients exhibited significantly higher expression of CD69%, CD38% CD69/19%, CD38/19% and CD38/CD69% than controls. High Expression of CD69 was positively correlated to poor prognostic features in our patients i.e., age, modified Rai staging, absolute lymphocytic count, B2 microglobulin, LDH, Coombs' test, receiving chemotherapy, CD38 expression and Thymidine kinase. There was significant higher expression of CD69% in patients who received chemotherapy, had LDT <12 months, and had positive Coombs's test. Focusing on patients who started chemotherapy, we found that they positively express CD69 than those who were untreated. These findings are consistent with **Del Poeta *et al.*** [22] who stated that CD69 is substantially connected with poor clinical predictive factors and could be thought of as an independent illness predictive marker. CD69 positive CLL patients got chemotherapy was the predominant category, and low CD69 expression was necessary to predict the longer progression free survival. In research done at Egypt on 153 (B-CLL) patients enrolled in Mansoura Oncology Center, **Aref *et al.*** [23] stated in accordance with these results that advanced Ria stage and greater B2M level were related to higher CD69% (2). Additionally, another study conducted on 40 B-CLL patients from Ain Shams University Hospitals, demonstrated highly significant elevation of CD69% expression between their studied groups with highly significant positive correlation between CD69% and hepatomegaly, Rai stage and CD38% expression. Furthermore, **Grywalska *et al.*** [24] found that greater Rai stages are linked to a greater proportion of CD19+CD69+ lymphocyte B, both in peripheral blood and bone marrow, and that there is a positive correlation between the LDH and CD19+CD69+% B lymphocyte. This research examined the expression and predictive role of CD25 and CD69 on the surface of T and B lymphocytes in CLL patients. They added that individuals who needed to start therapy because their illness was progressing quickly had more CD19+CD69+ B lymphocytes than those who were left untreated.

As regards TK activity, it was substantially greater in our CLL group than controls and in patients who received treatment than those untreated. Moreover, it was positively correlated with CD38% and CD69 %. These findings are in line with other investigations that have shown the prognostic value of TK activity in CLL. They stated that TK is consistent with the biology of the disease and the Rai staging system, allowing for differentiation between the disease's indolent and aggressive forms serving as an independent predictive of the duration of the disease and may aid in the early identification of indolent CLL [25].

Pathological angiogenesis is critical to the development of both hematological and solid cancers, including CLL. The primary pro-angiogenic factor thought to be responsible for the pathophysiology of CLL is VEGF, which is involved in the activation and regulation of blood vessel growth, and the activation of a variety of other pro-angiogenic processes [26].

According to these findings, VEGF serum levels were statistically significantly greater in CLL patients than in controls. It confirms earlier observations about the involvement of VEGF in the pathophysiology of CLL. Association of increased level of VEGF with treated patients and its positive correlation with CD38 and CD69 expression signifies its role as a bad prognostic marker. These data are also observed by **Smolej *et al.*** [27].

In this investigation, we sought to determine the prognostic significance of CD69 in relation to TK and VEGF in CLL patients. We discovered that elevated expression of CD69% is connected to high levels of TK and VEGF and directly related to poor prognostic factors, including advanced disease stages, LDT 12 months, and high CD38%. We recommend establishment of new scoring model that involves these three parameters as we predict in the shadow of our data that this scoring system will greatly help clinicians pick up patients who will exhibit aggressive course and will need to start treatment. With the advantages of simplicity and applicability of measuring these parameters, this scoring system could be widely used to predicts the disease severity instead of

more tedious and expensive investigations like cytogenetics and molecular studies, which are used to evaluate del 11q or 17p or immunoglobulin heavy chain variable regions mutational status.

CONCLUSION

CD69 together with TK and VEGF could be considered as poor prognostic markers and can add a new option for development of new prognostic scoring system or evolution of new treatment strategies.

DECLARATIONS

Consent for publication: I attest that all authors agreed to submit the work.
Availability of data and material: Available
Competing interests: None
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