ANGIOGENESIS' RELATED POLYMORPHISM EMERGES AS PROGNOSTIC MARKER IN DE NOVO LARGE B-CELL LYMPHOMA PATIENTS

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BACKGROUND

Angiogenesis (AG), the process in which new vessels are formed from preexisting vasculature, is crucial for the growth and progression of diffuse large B cell lymphoma (DLBCL) [1]. The most important key regulator of AG is the vascular endothelial growth factor (VEGF), and its functions is mediated by binding to a specific receptor, the VEGFR2 [2]. Both proteins are encoded by polymorphic genes and single nucleotide polymorphisms (SNPs) determining changes in their expressions have been described.

One of the main problems hindering the therapeutic success in DLBCL patients is the heterogeneity of this tumor, not only in clinical and morphologic level but also in the genetic level [3]. Immunohistochemistry (IHC) based on the expression of proteins that are differentially expressed between GCB and non-GCB were able to predict outcome of DLBCL patients [4, 5]. However, patients who present the same clinical features, when stratified by the Ann Arbor System, R-IPi or cell of origin IHC-based algorithm, may present distinct behavior, what suggests that new prognostic (genetic and IHQ markers) remains to be identified in disease.

Once that the association of *VEGF* 2578C/A (rs699947), 2489C/T (rs1005230), 1154G/A (rs1570360), 634G/C (rs2010963), 460C/T (rs833061) and 936C/T (rs3025039) SNPs, and *VEGFR2* 271G/A (rs7667298), 604T/C (rs2071559), 1192G/A (rs2305948) and 1719A/T (rs1870377) SNPs in tumor vasculature, VEGF/VEGFR2 expression, clinical features and survival of DLBCL patients is not yet clarified, the aim of this study was defined.

SUBJECTS AND METHODS

This prospective study included 168 consecutive *de novo* DLBCL patients seen at diagnosis at the Haematology and Haemotherapy Centre and A. C. Camargo Cancer Centre from June 2009 to September 2014. DLBCL was diagnosed according to the WHO classification and staged by Ann Arbor criteria. All patients were treated with conventional chemotherapy, including rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) [6]. All procedures were carried out according to the principles of the local ethics committee, and all subjects provided written informed consent.

Immunohistochemical analysis

Patients' diagnostic paraffin blocks served for IHC analyses using anti-CD34, anti-VEGF and anti-VEGFR2 antibodies. The slides were scanned at 20x magnification in Aperio Scanscope XT (Aperio Technologies, Vista, CA, USA) and submitted for digital analysis in a blinded fashion. Concerning CD34, microvessel density, vessel area (VA) and vascular perimeter (VP) were analyzed. VEGF was analyzed by the intensity of positivity (positive-stained pixels) and VEGFR2 was categorized in negative and positive cases.

Genetic polymorphism analysis

DNA from patients' peripheral blood samples was analyzed by real-time polymerase chain reaction (RT-PCR), using TaqMan® SNP Genotyping Assays from Applied Biosystems® (Carlsbad, CA, USA), for *VEGF* 2578C/A, 2489C/T, 1154G/A, 460C/T, 634G/C and 936C/T SNPs and *VEGFR2* 271G/A, 604T/C, 1192G/A and 1719A/T SNPs. All reactions were performed according to the manufacturers' instructions. The amount of 15% of genotype determinations was carried out twice in independent experiments with 100% of concordance.

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) was evaluated using the chi-square (χ^2) goodness-of-fit test. Haploview 4.2 software (www.broad.mit.edu/mpg/haploview) was used to tag all common haplotypes and their frequencies; *VEGF* and *VEGFR2* haplotypes with frequency higher than 10% were selected to analysis.

Differences between groups were analyzed by the χ^2 or Fisher's exact test. Two-tailed t-tests were performed to compare IHC scores between groups of patients. Logistic regression models assessed associations between genotypes and clinicopathological features.

Event-free survival (EFS) and overall survival (OS) were calculated from the date of diagnosis to date of progression, relapse, death by disease or last follow-up, and from the date of diagnosis to date of death for any reason or last follow-up, respectively. EFS and OS probabilities were estimated by Kaplan–Meier method and compared by log-rank test. The Cox hazards model was used to identify variables predicting EFS and OS. Variables with $P < 0.10$ in univariate Cox analysis were included in multivariate Cox analysis. Significant results were validated using a bootstrap resampling study to investigate the stability of risk estimates (1000 replications). Differences were significant when $P < 0.05$.

RESULTS

The patient's characteristics are presented in **Table 1**. The median of observation of patients enrolled in the study was 51 months (range: 1–117 months).

Concerning AG's SNPs and clinical features, *VEGF* 634GG was more frequent in stage III or IV patients (48.9% vs. 31.1%, $P = 0.02$) and *VEGF* 634CC genotype was more common in those who presented grade III or IV toxicities during treatment (21.6% vs. 8.5%, $P = 0.03$) when compared to others. *VEGF* 936CT or TT genotypes were more frequent in patients that did not achieve complete response (CR) (47.8% vs. 22.6%, $P = 0.01$) and was more common in patients with III or IV toxicity during treatment (41.2% vs. 19.8%, $P = 0.007$) when compared to others. *VEGFR2* 604TT was more frequent in poor R-IPi patients (43.7% vs. 27.8%, $P = 0.04$) when compared to others.

Concerning IHQ analysis, *VEGF* 634G allele was associated with higher vessel area and vascular perimeter; *VEGF* 936T allele was associated with *VEGFR2* expression and *VEGFR2* 1719AA genotype was associated with *VEGF* expression.

The prognostic impact of *VEGF* 1154 G/A SNP and *VEGF* ATCAGC haplotype is represented in Kaplan Meier curve (**Figure 1**) and Cox analysis (**Table 2**).

Table 1. Features of 168 diffuse large B-cell patients at diagnosis

Variable	N (%)
Age	
Median (range) in years	57 (19-89)
Gender	
Female	94 (55.9)
Male	74 (44.1)
B symptoms	
Absent	49 (29.2)
Present	119 (70.8)
Bulky disease	
No	111 (66.1)
Yes	57 (33.9)
Ann Arbor	
I+II	74 (44.1)
III+IV	94 (55.9)
R-IPi	
Very good/good	97 (57.7)
Poor	71 (42.3)
IHC-based subtype*	
GCB	88 (71.5)
Non-GCB	35 (28.5)
Response to R-CHOP**	
Complete	115 (83.3)
Partial	7 (5.1)
Stable or progressive disease	16 (11.6)
Toxicity grade III or IV***	
Haematological	49 (96.1)
Non-hematological	2 (3.9)

R-IPi: International Prognostic Index in rituximab era; IHC: Immuno-histochemistry; GCB: germinal center B-cells; *: IHC was performed only in patients with adequate lymph node tissue; **: Response to therapy was analyzed only in patients who completed the R-CHOP scheme; ***: toxicity was analyzed only in patients submitted at least for one cycle of R-CHOP

Table 2. Association of clinicopathological features and *VEGF* and *VEGFR2* genotypes with survival in diffuse large B-cell lymphoma patients

Characteristics	N event/ N total	Event Free Survival				N event/ N total	Overall Survival			
		Univariate HR (95% CI)	P value	Multivariate HR (95% CI)	P value		Univariate HR (95% CI)	P value	Multivariate HR (95% CI)	P value
Stage (Ann Arbor)										
I/II	25/74	Reference		Reference		20/74	Reference		Reference	
III/IV	45/94	1.66 (1.02-2.71)	0.04	NA	NA	41/94	1.86 (1.09-3.18)	0.02	NA	NA
B symptoms										
Absent	11/49	Reference		Reference		8/49	Reference		Reference	
Present	59/119	2.90 (1.52-5.52)	0.001	2.69 (1.23-5.86)	0.01*	53/119	3.49 (1.65-7.34)	0.001	2.92 (1.21-7.00)	0.01*
Histological subtype*										
GCB	30/88	Reference		Reference		27/88	Reference		Reference	
Non-GCB	22/35	2.31 (1.33-4.02)	0.003	2.67 (1.49-4.76)	0.001*	18/35	2.03 (1.11-3.71)	0.02	2.62 (1.41-4.86)	0.002*
R-IPi										
L/LI	29/97	Reference		Reference		22/97	Reference		Reference	
Poor	41/71	2.54 (1.57-4.09)	0.001	1.88 (1.04-3.41)	0.03*	39/71	3.12 (1.85-5.27)	0.0001	2.73 (1.43-5.19)	0.002*
VEGF1154										
GG	35/102	Reference		Reference		31/102	Reference		Reference	
GA + AA	35/66	1.83 (1.14-2.93)	0.01	2.08 (1.12-3.86)	0.01*	30/66	1.74 (1.05-2.88)	0.03	2.64 (1.41-4.91)	0.002*
VEGF936 CT + TT										
CC	45/124	Reference		Reference		41/124	Reference		Reference	
CT + TT	25/44	1.86 (1.13-3.04)	0.01	1.60 (0.86-3.00)	0.13	20/44	1.57 (0.91-2.68)	0.10	NA	NA
VEGFR21719										
AA	5/5	3.36 (1.34-8.36)	0.009	3.17 (0.97-10.21)	0.06	4/5	2.54 (0.92-7.02)	0.07	2.12 (0.61-7.36)	0.23
AT + TT	65/163	Reference		Reference		57/163	Reference		Reference	
VEGFATCAGC										
Absent	37/83	Reference		Reference		31/83	Reference		Reference	
Present	33/85	1.77 (1.11-2.84)	0.01	2.29 (1.27-4.11)	0.006*	30/85	1.78 (1.08-2.96)	0.02	2.69 (1.44-5.01)	0.002*

HR: Hazard ratio; CI: Confidence interval; NA: Not applicable; GCB: Germinal center B-cell; R-IPi, International Prognostic Index in rituximab Era. Ann Arbor stage was not included in multivariate analysis to avoid redundancy, since it was included in R-IPi. P values < 0.05 were considered significant and are presented in bold letters. ^(a) Pbootstrap= 0.003; ^(b) Pbootstrap= 0.004; ^(c) Pbootstrap= 0.004; ^(d) Pbootstrap= 0.05; ^(e) Pbootstrap= 0.04; ^(f) Pbootstrap= 0.001; ^(g) Pbootstrap= 0.004; ^(h) Pbootstrap= 0.002; ⁽ⁱ⁾ Pbootstrap= 0.003; ^(j) Pbootstrap= 0.005; ^(k) Pbootstrap= 0.006

Figure 1. a) Event-free survival (EFS) and b) overall survival (OS) of diffuse large B-cell lymphoma (DLBCL) patients stratified by genotypes of *VEGF* 1154G/A single nucleotide polymorphism (SNP); c) EFS and d) OS of DLBCL patients stratified by the presence *VEGF*ATCAGC haplotype

CONCLUSIONS

In conclusion, our data present evidence that inherited abnormalities in *VEGF* and *VEGFR2* SNPs, enrolled in AG pathway, are associated with prognosis in DLBCL. We believe that these results should be validated in study with larger sample size and by functional analyses of SNPs in tumor AG.

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