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*SEPT*10 Expression in Chronic Lymphocytic Leukemia. Correlation with Clinical and Biological Prognostic Factors

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is characterized by a highly variable clinical course. Microarray studies allowed highlight genes differentially expressed in this pathology. In this study, we have evaluated the prognostic significance of *SEPT*10 expression in CLL patients. Results were correlated with immunoglobulin heavy-chain variable (*IGHV*) genes mutational status, genomic rearrangements and clinical parameters. *SEPT*10 mRNA levels were determined by quantitative real-time PCR in 70 newly diagnosed CLL patients consecutively referred to our Institution. A wide heterogeneity for *SEPT*10 expression was found. Gene upregulation was observed in 18.5% of cases. The univariate analysis showed a positive association between gen expression and platelet count ($p < 0.0001$) and a negative correlation with hemoglobin levels ($p = 0.0094$). Although no significant differences were observed, mean treatment free survival was shorter in patients with high expression (31 months) with respect to those with low mRNA levels (72 months). Cases with abnormal karyotypes had increased expression compared to those with normal karyotypes and no association between gene expression and FISH (fluorescence *in situ* hybridization) risk groups and *IGHV* mutational status was found. Cases using *IGHV*3-23 gene rearrangement had low *SEPT*10 expression. Our results showed an association between *SEPT*10 expression and features of adverse outcome but without independent prognostic value. The study of *SEPT*10 expression may be important for a better understanding of disease heterogeneity, adding further information to those provided by established prognostic factors.

Keywords: Chronic Lymphocytic Leukemia; *SEPT*10 Expression; FISH; Cytogenetics; *IGHV* Mutational Status

1. Introduction

Chronic lymphocytic leukemia (CLL) is the most frequent type of adult leukemia in Western world. The disease is characterized by a highly variable clinical course, with time to progression ranging from months to decades [1]. Clinical staging systems have been very useful in guiding disease management and treatment decisions. However, these staging systems have shown a limited capacity to predict clinical outcome at an early stage of the disease. Based on this situation, a variety of biomarkers have been investigated as potential prognostic factors in CLL. They include genomic aberrations, mutational status of immunoglobulin heavy-chain variable (*IGHV*) genes and expression profiles of different genes. Particularly, fluorescence in situ hybridization (FISH) analysis allowed the identification of distinct cytogenetic risk groups, in which patients with deletion 13q14 as a single alteration have a better outcome, while patients with deletions 11q22 or 17p13 show the shortest median survival and, cases with trisomy 12 have an intermediate prognosis [2]. In addition, *IGHV* defines two disease subgroups: one expressing mutated (M) *IGHV* segments with a more favorable clinical course, and the other expressing unmutated (UM) *IGHV* segments, associated to a poor outcome [3,4].

More recently, microarray studies allowed the establishment of a gene expression profile characteristic of CLL which highlights differentially expressed genes in patients with stable or progressive disease. Among them, *SEPT*10 (septin 10), located at 2q13 band, has been proposed as a probable prognostic marker in CLL. This gene belongs to septins family, an evolutionarily conserved group of at least 14 distinct genes encoding GTP (gua-

nosine triphosphate)-binding proteins and filament-forming proteins, involved in multiple cellular processes like vesicle trafficking, apoptosis, remodeling of the cytoskeleton, infection, neurodegeneration and neoplasia [5]. Many evidences have indicated that some septins were abnormally expressed in certain kinds of tumors and the altered expressions were related to the process of carcinogenesis [6]. In addition, mammalian septins modulate microtubule dynamics through the interaction with a microtubule associated protein, MAP4 [7], and particularly *SEPT*10 is an important regulator of micro-tubule stability [8]. In CLL, the literature showed that *SEPT*10 mRNA expression correlates with both high lipoprotein lipase (*LPL*) expression and UM *IGHV* gene status [9,10]. However, its association with other characteristics of the disease has been scarcely explored. In this study we have evaluated the prognostic significance of *SEPT*10 expression in CLL patients in relation to *IGHV* mutational status, genomic rearrangements and clinical parameters.

2. Material and Methods

2.1. Patients

Our cohort included 70 newly diagnosed CLL patients (43 males; median age: 65 years, range: 38 - 83 years) consecutively referred to our Institution. Patients were diagnosed according to the International Workshop on Chronic Lymphocytic Leukemia criteria [11]. Stage was assessed according to the Rai classification [12]. Clinical stages showed the following distribution: 0: 25 patients (36%), I: 10 (14%), II: 22 (31%), III: 7 (10%) and, IV: 6 (9%). All individuals provided their informed consent. The study was approved by the local Ethics Committee.

2.2. Cytogenetic and FISH Analysis

Chromosome analysis was performed on peripheral blood lymphocytes, cultured for 96 hours at 37˚C in F-12 medium supplemented with 15% of fetal calf serum, stimulated with Pokeweed mitogen and Lipopolysaccharide, or in presence of the immunostimulatory CpG-oligonucleotide (2 μM) and interleukin 2 (200 U/ml) for 72 hs. Slides were prepared by conventional method. G-banding technique was used.

For FISH analysis, slides were hybridized with LSI TP53/ATM/13q14/13q34/CEP12 DNA probes (Vysis-Abbott), according to manufacturer's protocol. Two hundred interphase nuclei were analyzed for each probe. The cut-off for positive values (mean of normal control + 3 standard deviations), determined from samples of ten cytogenetically normal donors, were: 3.02%, 10.2%, 7.7% and 5.1% for trisomy 12, monosomies of D13S319 (13q14), ATM (11q22) and TP53 (17p13), respectively.

2.3. mRNA Extraction and *SEPT*10 Expression Analysis

Total RNA was extracted with Trizol reagent (Invitrogen, Buenos Aires, Argentina) from mononuclear cells isolated on a Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Uppsala, Sweden) density gradient from peripheral blood samples of patients and healthy controls. The cDNA synthesis was carried out using Moloney Murine Leukemia Virus Reverse Transcriptase and random primer (Promega, Madison, WI, USA). *SEPT*10 mRNA levels were determined by quantitative real-time PCR (qRT-PCR) in a LightCycler® 2.0 system (Roche Diagnostics, Mannheim, Germany), based on SYBR Green I methodology, and utilizing the Lightcycler software. Briefly, 1 μg of total RNA was amplified in the presence of 0.5 μM of specific primers for *SEPT*10 [10] or the housekeeping gene *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) [13] with the following protocol: 10 min at 95˚C for initial denaturation, 50 cycles of 15 s at 95˚C, 10 s at 62˚C and 15 s at 72˚C, followed by the respective *melting* curve. All measurements included a determination of the standards and the use of a no-template as a negative control.

2.4. *IGHV* Mutational Status

The *IGHV* gene sequences were determined as previously described [14]. Briefly, amplification of *IGHV* regions by PCR was performed on cDNA using VH framework region 1 consensus family specific primers (VH1-VH6) and JH primers [15]. When amplifications of these primers were unsuccessful, an alternative set of primers that anneal to sequences in the leader region (LH1-LH6) and one antisense Cμ-primer [16] were used. Thermal cycling conditions were 3 min at 93˚C, followed by 33 cycles at 94˚C for 30 s, 62˚C for 30 s, 72˚C for 30 s, elongation at 72˚C for 7 min, and a final step at 4˚C for 10 min. PCR products were purified in 1% agarose gels, sequenced bi-directionally and analyzed on an automated DNA sequence analyzer (377 ABI Prism, PE Biosystem, Foster City, CA). Sequence data were analyzed using IgBLAST (http://www.ncbi.nlm.nih.gov/igblast) and the ImMunoGeneTics database (IMGT; http://imgt.cines.fr). *IGHV* sequences with less than 98% homology with respect to the germline counterpart were considered as M while those with homology of 98% or higher were classified as UM [3,4].

2.5. Statistical Analysis

Statistical evaluation of mRNA expression data was performed using the Mann-Whitney test. Correlations between gene expression and clinical variables were assessed by using the Kendall's coefficient. The cut-off

point for *SEPT*10 expression was selected according to receiver operating characteristic (ROC) analysis. Group wise comparison of the distribution of clinical and laboratory variables was performed with the Student t test (for quantitative variables) and the χ^2 or Fisher's exact test (for categorical variables). Overall survival and treatment free survival (TFS) were estimated by the Kaplan-Meier method and compared with the log-rank test.

3. Results

Analysis of data showed a wide heterogeneity for *SEPT*10 expression in CLL patients, with cases that overexpressed this gene and others with low levels of transcripts (**Figure 1**). For a better analysis, patients were divided into two groups: high expression (*SEPT*10^{high}) and low expression (*SEPT*10^{low}), according to the cut-off value (221.05) obtained by ROC curves. It was quite similar to the baseline value of *SEPT*10 expression observed in control samples with our approach (220.3 ± 107.5). Upregulation of *SEPT*10 was found in 13 cases (18.5%) (mean ± SE: 720.9 ± 190.5) while the remaining patients showed low levels of mRNA transcript (mean ± SE: 44.26 ± 6.99).

Furthermore, we would like to determine if *SEPT*10 expression profile had clinical significance. The analysis according to established prognostic factors in CLL did not show differences between patients with high and low gene expression, with the exception of age, that was reduced in *SEPT*10^{low} patients compared to those with high mRNA levels ($p = 0.006$) (**Table 1**). The univariate analysis showed a positive association between gen expression and platelet count ($p < 0.0001$) and a negative correlation with hemoglobin levels ($p = 0.0094$). In reference to outcome, although no significant differences were observed, mean TFS was shorter in *SEPT*10^{high} patients (31 months) with respect to those with low gene expression (72 months).

We also analyzed *SEPT*10 expression in relation to cytogenetic alterations, FISH risk groups and *IGHV* mutational status, and a similar distribution of patients in the different categories was observed (**Table 2**). However, when *SEPT*10 mRNA levels were evaluated, patients with abnormal karyotypes had increased expression compared to those with normal karyotypes (**Figure 2**) and no association between gene expression and FISH risk groups and *IGHV* mutational status were found. Expression levels were also evaluated in different *IGHV* families and a similar distribution among VH1, VH3 and VH4 was observed. More detailed analysis of *IGHV* segments usage showed that *IGHV*3-23 gene rearrangement was the most represented in our cohort (7 cases); six of them (85.7%) showing low *SEPT10* expression, as well as the only one case of our series using *IGHV*3-21 segment.

4. Discussion

CLL is characterized by a heterogeneous clinical course, with some patients experiencing rapid disease progression and others living for decades without requiring treatment. However, classical prognostic parameters have a limited capacity to predict clinical outcome at an early stage of the disease. In recent years, numerous genetic approaches have provided new markers for prognosis and response prediction [17]. Among them, a considerable number of genes differentially expressed in CLL were identified, including *SEPT*10 [18,19], which role as prognostic factor in this pathology was scarcely evaluated [9,10]. In this study, we have analyzed *SEPT*10 mRNA expression in a cohort of newly diagnosed CLL patients. Our series showed a low percentage of patients with *SEPT*10 overexpression (18% of cases), value close to the 27% observed by Van't Veer *et al*. [10], indicating that deregulation of this gene is limited to a specific subgroup of patients. Besides, expression analysis showed a wide heterogeneity of mRNA levels, which could be related to variations in expression control at the transcripttional, post-transcriptional and translational levels or

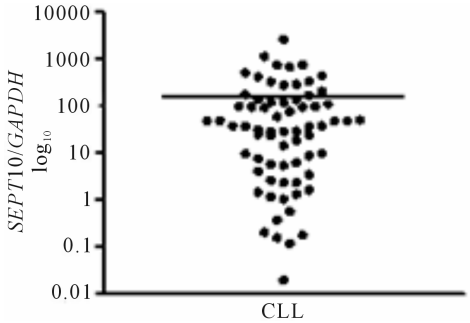

Figure 1. Scatter plot showing the wide distribution of *SEPT*10 mRNA levels in chronic lymphocytic leukemia (CLL) patients.

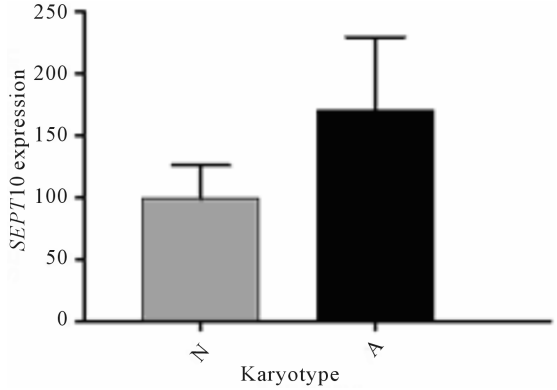

Figure 2. *SEPT*10 mRNA expression profile in chronic lymphocytic leukemia patients with normal (N) and abnormal (A) karyotypes.

Table 1. *SEPT*10 gene expression according to the known prognostic factors in CLL.

Expression groups (No. cases)	Mean age (years)	Sex (%)		RAI stages (%)			Mean % lymphocytes (range)	Mean platelet count ($\times 10^9$/L) (range)	Mean Hb (g/dL) (range)	Mean LDH (UI/L) (range)	Mean β2M (μg/ml) (range)
		M	F	0	I - II	III - IV					
*SEPT*10^{low} **< 221.5 (57)**	61.7	65	35	31.8	47.7	20.5	75.5 (49 - 98)	192.5 (33 - 378)	12.9 (7.4 - 15.5)	383 (120 - 680)	2.6 (1.1 - 3.6)
*SEPT*10^{high} **≥ 221.5 (13)**	71.4[a]	50	50	54.5	36.4	9.1	66.2 (54 - 87)	273.1 (76 - 900)	12.2 (5.6 - 15.8)	418 (269 - 497)	2.5 (1.2 - 3.6)

M: male; F: female; Hb: hemoglobine; LDH: lactate dehidrogenase; β2M: beta 2 microglobuline. [a]Significant differences respect to *SEPT*10^{low} patients: p = 0.006.

Table 2. *SEPT*10 gene expression according to cytogenetic, FISH and *IGHV* groups.

Expression groups (No. of cases)	Cytogenetics (%)		FISH (%)		*IGHV* (%)	
	NK	AK	NF	AF	M	UM
***SEPT*10^{low} < 221.5 (57)**	72.5	27.5	71.5	28.5	55.6	44.4
***SEPT*10^{high} ≥ 221.5 (13)**	66.7	33.3	77.7	22.3	75.0	25.0

NK: normal karyotype; AK: abnormal karyotype; NF: normal FISH; AF: abnormal FISH; *IGHV*: immunoglobulin heavy-chain variable gene; M: mutated; UM: unmutated.

associated to the presence of alternative splice variants [5]. Citoquines or mitogens that induce proliferation and differentiation through different signaling pathways may also influence the activity of this gene.

As previously referred, clinical significance of *SEPT*10 mRNA expression was scarcely evaluated in the literature [9,10,20]. In this aspect, our results add new information about it. Thus, we found association between *SEPT*10 expression and age, platelet count and hemoglobin levels. In addition, although no significant differences were found, longer TFS was observed in our *SEPT*10^{low} subgroup, supporting a previous report showing that patients with high *SEPT*10 expression required earlier treatment [9]. Simultaneously, we have evaluated for the first time the association between cytogenetic alterations and *SEPT*10 expression and cases with abnormal karyotypes had a non-significant *SEPT*10 upregulation compared to those with normal karyotypes. These findings are of interest taking into account the adverse clinical impact of structural abnormalities and complex karyotypes in CLL [21-23], suggesting a relationship between *SEPT*10 expression and accumulation of chromosome alterations in leukemic cells. In addition, we did not observe correlations between *SEPT*10 levels and FISH risk groups, coincident with the sole report in the literature that analyzed this aspect [10].

Unlike to those observed in the literature [9,10,20], we did not find association between *SEPT*10 expression and *IGHV* mutational status. A study in a larger series may be necessary to clarify this point. In reference to *IGHV* segment usage, association between low *SEPT*10 mRNA levels and *IGHV*4-34 and high expression with *IGHV*1-69 gene rearrangement, were reported [10]. Although the number of our cases is not large, an association between *IGHV*3-23 and low expression was observed. Interestingly, different authors [10,24] have detected lower *SEPT*10 expression in *IGHV*3-21 expressing CLL, suggesting the inability of *SEPT*10 expression to recognize the poor prognosis of this CLL subset. The only one case of our series expressing *IGHV*3-21 segment supports these findings.

In conclusion, our results showed a wide heterogeneity in the expression profile of *SEPT*10 with overexpression in a subgroup of CLL patients. The correlation with clinical characteristics showed association with features of adverse outcome but without independent prognostic value. Thus, the study of this gene may be important for a better understanding of the disease variability, and could be used in combination with other markers, adding further information to those provided by established prognostic factors. In this line, an association between *SEPT*10 and *LPL* [9], which overexpression was related to adverse clinical outcome in CLL [25], have been previously described, suggesting that *SEPT*10 could direct *LPL* to the cell surface by establishing a link between proteoglycans (the cell surface *LPL* receptor) and the actin cytoskeleton [5,9]. The understanding of biological mechanism of the disease will contribute to future risk stratification strategies with potential value in treatment decisions.

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