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ORIGINAL ARTICLE

A proliferation-inducing ligand (APRIL) serum levels predict time to first treatment in patients affected by B-cell chronic lymphocytic leukemia

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Abstract

Purpose: A proliferation-inducing ligand (APRIL), a tumor necrosis factor superfamily member involved in B-lymphocytes differentiation and survival, plays a role in protecting B-Cell Chronic lymphocytic leukemia (B-CLL) cells from apoptosis. Having observed that APRIL serum (sAPRIL) levels were higher in B-CLL patients with CLL at diagnosis as compared to healthy donors (14.61 ± 32.65 vs. 4.19 ± 3.42 ng/mL; $P < 0.001$), we tested the correlation existing in these patients between sAPRIL, clinical–biological parameters and disease progression. Experimental design: sAPRIL levels were measured by ELISA in 130 patients with B-CLL at diagnosis and in 25 healthy donors. Results: sAPRIL levels did not correlate with gender, age, clinical stage, blood cell counts, β2-microglobulin (β2M) levels, ZAP-70 and CD38 expression. Using median sAPRIL natural logarithm (ln) as cutoff, we distinguished two groups of patients ($APRIL_{LOW}$ and $APRIL_{HIGH}$) who were comparable with regard to clinical–biological parameters and overall survival, but different with regard to time to the first treatment (TTFT; $P = 0.035$). According to univariate analysis, high lymphocyte count, high β2M, Binet stage B–C, ZAP-70 expression and ln(sAPRIL) above median were associated with earlier TTFT. Advanced clinical stage, high β2M, ZAP-70 expression and ln(sAPRIL) above median remained independently predictive of shorter TTFT at multivariate analysis. Moreover, sAPRIL increased its prognostic significance when patients were stratified according to independent favorable clinical–biological characteristics (low β2M, stage A and lack of ZAP-70 expression). Conclusions: sAPRIL is a novel indicator of shorter TTFT in B-CLL and a predictor of progression especially in patients otherwise considered at low risk according to validated prognostic factors.

Key words a proliferation-inducing ligand; time to first treatment; B-cell chronic lymphocytic leukemia

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The tumor necrosis factor (TNF) superfamily member a proliferation-inducing ligand (APRIL) has been shown to play an important role in B-cell biology (1, 2). *In vitro*, APRIL drives both proliferation and survival of human and murine B cells (1), while *in vivo*, it induces in mice a spleen enlargement because of infiltration by CD5-positive B lymphocytes (3). APRIL expression has been reported, under physiological conditions, in several hematopoietic cells (neutrophils, monocytes, macrophages, dendritic cells, B and T lymphocytes and osteoclasts) (2). It has also been detected in solid tumors and in B-cell-derived malignancies (2). Initially produced as a homotrimeric type-II transmembrane protein, APRIL is mainly released in the extracellular compartment as a soluble form (4). It interacts with two canonical receptors, the B-cell maturation antigen (BCMA) and the transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) (2). Furthermore, it has been shown to react also with the heparan sulfate side chain of proteoglycans (HSPG), which is supposed to

facilitate TACI and/or BCMA signaling (5). Several studies have reported the involvement of APRIL in neoplastic diseases (6), especially in B-cell-derived malignancies (2). *In vitro* studies have demonstrated that APRIL induces the proliferation of human follicular lymphoma B cells (7), promotes the survival of diffuse large B-cell lymphoma cells (8), the survival and proliferation of Hodgkin's lymphoma cell lines (9) and, finally, protects from apoptosis dexamethasone-treated human multiple myeloma cells (10). Additional studies have indirectly indicated a role of APRIL in the pathogenesis of high-grade non-Hodgkin's (11) and Hodgkin's lymphomas (12). Increasing lines of evidence are showing that APRIL is also involved in the pathogenesis of B-cell chronic lymphocytic leukemia (B-CLL), which is characterized by the progressive accumulation of mature and apoptosis-resistant CD5-positive B lymphocytes in lymphoid tissues, bone marrow (BM) and peripheral blood. Recent reports have demonstrated that the majority of B-CLL cells express detectable levels of BCMA and TACI on their surface (13, 14) and that malignant lymphocytes themselves may express and release APRIL (15). Other reports have indicated in nurse-like cells differentiating from CD14-positive cells the main source of APRIL in B-CLL (16). *In vitro,* treatment of B-CLL cells with recombinant human APRIL (rhAPRIL) has been shown to promote B-CLL cells survival through the activation of the NF-kB pathway (13). Interestingly, approximately 40% of aged human APRIL transgenic mice develop a B1-cell-associated neoplasia resembling B-CLL (2).

On the basis of these experimental data, we aimed to establish the impact of APRIL serum (sAPRIL) levels on the clinical outcome of patients with B-CLL, assessed by Cox's proportional hazard modeling in both the univariate and multivariate analyses. To pursue this aim, we retrospectively evaluated 130 patients observed in our Institution, studying the correlation between sAPRIL levels and the time elapsing from the diagnosis to the first treatment (TTFT). According to our study, increased levels of sAPRIL appeared predictive of a shorter TTFT independently from other prognostic factors such as high lymphocyte count, high β2M, advanced clinical stage and ZAP-70 expression. This also applied when a subgroup of low β2M, early-stage, ZAP-70-negative patients were separately evaluated, further confirming the potential clinical value of sAPRIL measurement in B-CLL.

Patients and methods

Patients

We retrospectively evaluated 130 patients diagnosed with B-CLL between November 1993 and March 2009 and followed from then on at our Institution. Diagnosis was based or reviewed according to the most recent published criteria (17, 18), and the patients were selected only on the basis of availability of sera collected at diagnosis and immediately frozen. This series encompasses 35% of the whole B-CLL population diagnosed by our Institution in the aforementioned period. Informed consent was obtained according to the legal regulation present in Italy at the time of diagnosis, and, in the case of recent samples, it was provided according to the Declaration of Helsinki for the collection and use of biological samples for Institutional Review Board (IRB)-approved research purposes. Patient's evaluation included history, physical examination, laboratory evaluation (complete blood count and chemistries), peripheral blood smear morphology and BM aspirate and biopsy. Clinical stage was measured according to Binet scale. On the basis of the equation devised by Delgado and coll (19), β2M levels, available in 108 patients (83%), were adjusted according to patient's individual glomerular filtration rate (GFR): GFR-β2M = β2M (mg/L) $\times$ GFR (mL/min)/100. Cutoff level was established at 2 mg/L (19). CD38 expression was evaluated in peripheral blood from 105 patients (81%) by flow-cytometry (cutoff: 30% of B lymphocytes). ZAP-70 expression was evaluated in BM specimens from 116 patients (89%) by immunohistochemistry (IHC). The reliability of this technique and its correlation with immunoglobulin heavy chain (IgVH) gene mutational status has already been described in the literature (20, 21). Therefore, considering that IgVH mutational status was available only in the most recent cases of our series (43%) and that in our study we observed an 88% concordance rate between IgVH mutational status and ZAP-70 expression, we decided to use ZAP-70 as surrogate for statistical analysis. Data regarding FISH analysis, available only in a minority of patients (32%), were excluded from statistical analysis owing to the methodological reasons. Clinical and biological data of the entire series are summarized in Table 1.

APRIL measurement

sAPRIL levels were measured by ELISA (Bender MedSystem GmbH, Vienna, Austria), according to the Manufacturer's instructions in serum samples collected and frozen at diagnosis. No systematic bias has been introduced in deciding which sera to stock at diagnosis, nor in the freezing procedure. All the ELISAs have then been run between the end of 2009 and the beginning of 2010. Measurements were taken in triplets, and mean values were taken as final data. The calculated median intra-assay variability was 7.57% (range: 5.22–9.04), while inter-assay variability coefficient of variation was 9.2%.

Table 1 Patients clinical–biological characteristics and distribution according to median ln(sAPRIL) levels

	OVERALL	$APRIL_{LOW}$ GROUP	$APRIL_{HIGH}$ GROUP	*P*
Number of patients	130	72	58	
Median age, years (range)	64 (33–92)	62 (34–92)	66.5 (33–88)	NS
Male/Female gender	79/51	42/30	37/21	NS
Binet stage (A vs. B–C)	90/40	50/22	40/18	NS
Mean hemoglobin (g/dL) ±SD	13.5 ± 1.98	13.7 ± 1.53	13.3 ± 2.42	NS
Mean lymphocyte count ($\times 10^9$/L) ±SD	26.63 ± 33.70	21.16 ± 26.70	29.02 ± 40.72	NS
Mean platelet count ($\times 10^9$/L) ±SD	209.600 ± 103.9	198.167 ± 67.2	223.793 ± 135.9	NS
GFR-β2M[1] (mg/L) ±SD	2.27 ± 1.68	2.35 ± 2.02	2.17 ± 1.12	NS
CD38-positive cases[2]	61/105 (58.1%)	37/57 (64.9%)	24/48 (50.0%)	NS
ZAP-70-positive cases[3]	76/116 (65.5%)	41/63 (65.0%)	35/53 (66.0%)	NS
IgV_H gene mutational status[4]				
Unmutated	40 (67.8%)	24/40 (60.0%)	16/40 (40.0%)	NS
Mutated	19 (32.2%)	12/19 (63%)	7/19 (37.0%)	NS

Missing: 22 patients[1], 25 patients[2], 14 patients[3], 74 patients[4].

Statistical analysis

sAPRIL appeared as a log-normal variable in samples from healthy donors. As such, its natural logarithm followed a normal frequency distribution when tested by means of a Shapiro–Wilk test ($P = 0.464$), with gaussian symmetry around the median. Based on these results, we applied a logarithmic conversion to all sAPRIL data, and then, we used the median ln(sAPRIL) (=1.7851) as cutoff for significance to divide our series into two groups of statistically comparable size ($APRIL_{LOW}$, $n = 72$ and $APRIL_{HIGH}$, $N = 58$). These two groups were then compared according to their characteristics using Fisher's exact test and Pearson's chi-squared, for proportions, and independent Student's *t*-test, for parametric variables. Considering that the two groups were balanced for all clinical and biological parameters, we used the same cutoff – median ln(sAPRIL) – to assess the potential prognostic power of sAPRIL levels and for further statistical evaluation.

sAPRIL levels were then correlated with other numeric variables (age, hemoglobin, lymphocyte and platelet counts, GFR-β2M) by means of a linear regression [ordinary least squares method (OLS)], and the correlation of sAPRIL with categorical and binary variables was tested by means of the Mann–Whitney *U* test (between two groups) and the Kruskall–Wallis test (among multiple groups). ZAP-70 and CD38 expression by B-CLL cells were considered as binary variables, and correlation with sAPRIL levels was tested by the Mann–Whitney *U* test. Overall survival (OS) was defined as the time from diagnosis to death from all causes or to last follow-up, TTFT was defined as the time from diagnosis to any initial treatment or, alternatively, as time from diagnosis to last follow-up. Survival data were considered as per March 2010, when 80 patients were alive (61.5%) with a median follow-up of 88 months (range 12–195). Progression and indication to treatment were defined according to the National Cancer Institute-sponsored working group guidelines for chronic lymphocytic leukemia (17, 18). Briefly, criteria for initiating primary treatment were the following: (i) the presence of constitutional symptoms; (ii) evidence of progressive marrow failure as manifested by the development of, or worsening of, anemia and/or thrombocythemia; (iii) autoimmune anemia and/or thrombocytopenia poorly responsive to corticosteroid therapy; (iv) massive (>6 cm below the left costal margin) or progressive splenomegaly; (v) massive nodes or clusters (>10 cm in longest diameter) or progressive lymphadenopathy; (vi) progressive lymphocytosis with an increase of >50% over a 2-month period or an anticipated doubling time of <6 months (17, 18). In any case, absolute lymphocyte count was never used as the sole indicator for the treatment. Our Institution did not change the policy on indications to treatment over the years encompassed in the study.

OS and TTFT curves were plotted according to Kaplan–Meier's method, and differences between the groups were tested using the log-rank test (*P* value for significance = 0.05). The evaluation of putative risk factors was made using Cox's proportional hazard modeling to obtain hazard ratios (HR) and their 95% confidence intervals (95% CI), in both univariate and multivariate modes. All statistical calculations were carried out using Stata™ IC v.10.0 (StataCorp, College Station, TX, USA) for Microsoft Windows®.

Results

APRIL is significantly increased in patients with B-CLL

sAPRIL serum levels were significantly increased in patients with B-CLL as compared with those of age- and gender-matched healthy donors (mean ± SD, 14.61 ng/mL ± 32.65, $n = 130$ vs. 4.19 ± 3.42 ng/mL, $n = 25$, respectively: $P < 0.001$; Fig. 1). In patients with B-CLL,

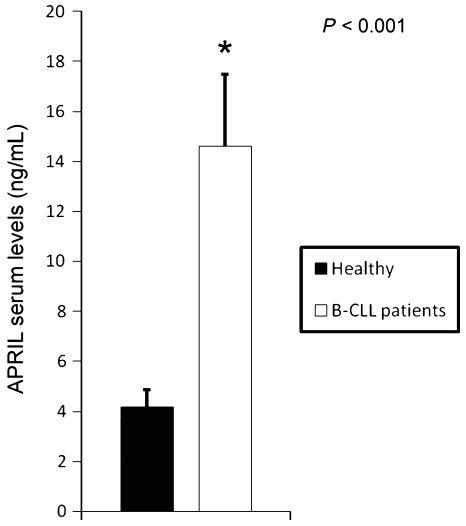

Figure 1 sAPRIL levels (ng/mL, mean ± standard deviation) in healthy donors (n = 25) and in patients with B-chronic lymphocytic leukemia (n = 130).

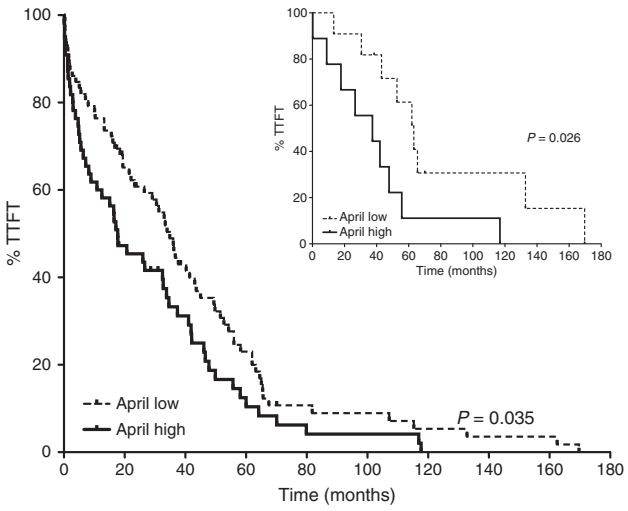

Figure 2 Time to the first treatment according to ln(sAPRIL) levels in the overall population and in a subgroup of patients (insect) characterized by favorable prognostic factors (β2M < 2 mg/L, stage A, lack of ZAP-70 expression).

sAPRIL did not appear to correlate with any of the following clinical–biological parameters: age, gender, clinical stage, hemoglobin levels, lymphocyte or platelet count, GFR-β2M, CD38 or ZAP70 (data available from the authors).

APRIL identifies patients at high risk of earlier progression

As described earlier, median ln(sAPRIL) (1.7851) was used as cutoff allowing to identify two groups of patients $APRIL_{LOW}$ (n = 72) and $APRIL_{HIGH}$ (n = 58), who were comparable regarding all clinical–biological parameters (Table 1). Pearson's chi-squared and Fisher's exact test showed no significant association between APRIL risk group and stage or ZAP-70 expression when all patients were considered. Furthermore, sAPRIL levels did not significantly differ between ZAP-70-positive and ZAP-70-negative patients in subgroups defined by clinical stage (A vs. B–C). Interestingly, when considering TTFT, the two groups of patients revealed a different outcome (Fig. 2). Indeed, median TTFT was 34 months in the $APRIL_{LOW}$ group and 17 months in the $APRIL_{HIGH}$ (P = 0.035). OS, on the contrary, was not significantly different in the two groups (median OS 122 vs. 144 months, P = NS).

APRIL is a predictor of shorter TTFT, independently from favorable prognostic factors

We evaluated prognostic value of age, gender, lymphocyte count at diagnosis, clinical stage, GFR-β2M > 2 mg/L, ZAP-70 and CD38 expression and median ln(sAPRIL), at both the univariate and multivariate levels. At the univariate analysis, a lymphocyte count at diagnosis $> 30 \times 10^9$/L, GFR-β2M > 2 mg/L, clinical stage B–C, ZAP-70 expression and ln(sAPRIL) above the median were significantly associated with earlier progression: GFR-β2M > 2 mg/L, stages B–C, ZAP-70 expression and ln(sAPRIL) above the median remained independently predictive also at a subsequent multivariate analysis (Table 2).

When we stratified our series according to stage, we found that ln(sAPRIL) above the median was able to predict earlier TTFT in patients in stage A (n = 90), with a median TTFT of 45 months in the $APRIL_{LOW}$ (n = 50) and 32 months in the $APRIL_{HIGH}$ (n = 40) group, respectively (P = 0.019). On the contrary, ln(sAPRIL) prognostic value was lost in advanced (B–C) stages (n = 40; P = NS). Significantly, ln(sAPRIL) was also a powerful predictor of earlier TTFT in ZAP-70-negative patients (n = 40), with a median TTFT of 53 months in $APRIL_{LOW}$ (n = 22) and 31 months in $APRIL_{HIGH}$ (n = 18) patients, respectively (P = 0.025). ln(sAPRIL) prognostic value was lost in the case of ZAP-70-positive patients (n = 76) (P = NS). Interestingly, in a relatively small group of patients (n = 20) characterized by all the independent favorable prognostic factors resulting from our multivariate analysis (GFR-β2M < 2mg/L, stage A, lack of ZAP-70 expression), ln(sAPRIL) above the median was still able to discriminate a subgroup of patients with a significant lower TTFT (P = 0.026; Fig. 2).

Table 2 Factors predictive of earlier time to the first treatment according to Cox's proportional hazard modeling

	Unadjusted HR (95% CI)	*P*	Adjusted HR (95% CI)	*P*
Age >65 yrs	1.29 (0.89–1.85)	NS		
Male gender	1.14 (0.79–1.65)	NS		
Lymphocyte count $>30 \times 10^9$/L	2.06 (1.31–3.22)	0.002	1.38 (0.80–2.36)	NS
Binet stage B–C	6.02 (3.71–9.77)	<0.001	4.38 (2.35–8.14)	<0.001
GFR-β2M ≥ 2mg/L	1.86 (1.25–2.76)	0.002	1.65 (1.02–2.66)	0.042
ZAP-70 expression	2.03 (1.30–3.17)	0.002	1.72 (1.01–2.92)	0.046
CD38 expression	1.49 (0.98–2.27)	NS		
sAPRIL ≥ median ln	1.48 (1.03–2.14)	0.036	1.81 (1.16–2.84)	0.010

GFR, glomerular filtration rate; HR, hazard ratios.

Discussion

The clinical course of patients with B-CLL is highly variable, with life expectancies ranging from months to several years. During the last decades, many efforts have been made to identify prognostic markers capable of discriminating high-risk patients who might benefit from more aggressive approaches. Nowadays, several validated prognostic markers are available (22), and the more recent efforts are aimed at identifying novel factors useful for refining the prognosis of risk categories identified by already existing markers. The involvement of sAPRIL in the pathogenesis of lymphoproliferative diseases prompted us to explore its prognostic role in the whole series of patients with B-CLL and particularly in well-defined risk categories. Previously, Planelles *et al*. (23) have demonstrated that among 95 patients affected by B-CLL, those with serum concentration of APRIL above median had significantly poorer OS. In that study, though, $APRIL^{high}$ group consisted mostly of patients with clinical unfavorable features, such as Binet B–C. In the other study available on this topic, Bojarska-Junal *et al*. (24) have analyzed APRIL plasma levels with regard to OS in a cohort of 183 patients with B-CLL, demonstrating that APRIL plasma levels above median correlated with more advanced (Rai 3–4) stages and predicted worse OS. The fact that also in this case, patients with high APRIL concentrations clustered in unfavorable or advanced stage groups may have partially accounted for their poorer OS. Differently from the two previous reports, we did not observe a predictive role of sAPRIL in OS, but in TTFT. In our opinion, this finding may be due to the fact that in our series, APRIL divided patients into two groups characterized by statistically comparable adverse clinical and biological features. Moreover, heterogeneous treatments adopted throughout the two decades covered by our study could also have impacted on the final outcome of patients (25). Unfortunately, no data on TTFT are available for comparison from the aforementioned studies (23, 24).

However, in the present report, we demonstrate that increased sAPRIL levels can independently predict a shorter TTFT and identify a subgroup of poor-risk patients otherwise characterized by independent favorable predictors (GFR-β2M < 2 mg/L, stage A, lack of ZAP-70 expression), according to the multivariate analysis of our series. Interestingly, the inverse correlation between increased sAPRIL levels and the time elapsing from the diagnosis to the first treatment, particularly in low-risk patients, suggests a role for sAPRIL in supporting the resistance to apoptosis of malignant B lymphocytes, independently of favorable intrinsic features. Furthermore, as we did not find any association between sAPRIL levels and number or biological characteristics of malignant B-lymphocytes, it is conceivable to hypothesize a release of sAPRIL from the microenvironment. As a matter of fact, an increasing number of studies are indicating a role of the microenvironment in promoting and stimulating the proliferation and survival of B-CLL lymphocytes through the release of soluble mediators (26). We are aware that, given the relatively small sample size and the retrospective nature of the study our observation should be prospectively validated in a larger cohort of patients with B-CLL, fully characterized according to the most significant prognostic factors including cytogenetics.

We believe that besides the speculative interest, our findings add relevance to sAPRIL as a prognostic indicator and possible therapeutic target, especially considering the availability of agents capable of interfering with APRIL and currently tested in the clinical setting (27, 28).

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Conflict of interest

The authors declare no competing financial interests.

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