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ORIGINAL REPORT

Rel A Is an Independent Biomarker of Clinical Outcome in Chronic Lymphocytic Leukemia

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ABSTRACT

Purpose

We recently demonstrated the biologic importance of the nuclear factor kappa B (NF-κB) subunit Rel A in chronic lymphocytic leukemia (CLL) and hypothesized that Rel A DNA binding would have prognostic significance in this disease.

Patients and Methods

Rel A DNA binding was quantified in nuclear extracts derived from 131 unselected CLL patient samples using a quantitative DNA-binding enzyme-linked immunosorbent assay–based method. We then investigated the ability of Rel A to predict for the requirement for treatment and survival and compared our findings with other established prognostic markers.

Results

Rel A DNA binding was strongly associated with advanced Binet stage ($P < .0001$) but did not correlate with immunoglobulin V_H (IgV_H) mutation status ($P = .25$), CD38 expression ($P = .87$), or zeta-chain–associated protein kinase 70 (ZAP-70) expression ($P = .55$). It was predictive of time to first treatment ($P = .02$) and time to subsequent treatment ($P = .0001$). In addition, Rel A was the most predictive marker of survival both from date of diagnosis (hazard ratio [HR], 9.1; $P = .01$) and date of entry into the study (HR, 3.9; $P = .05$) and retained prognostic significance in multivariate analysis for both time to first treatment and overall survival in the presence of Binet stage, IgV_H mutation status, CD38, and ZAP-70.

Conclusion

Rel A is an independent prognostic marker of survival in CLL and seems to have the unique capacity to predict the duration of response to therapy. Prospective assessment of Rel A as a marker of clinical outcome and as a therapeutic target are now warranted.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the commonest leukemia in the Western world, but it manifests a heterogeneous clinical course.[1,2] Some patients show rapid disease progression with a mean survival of less than 36 months, whereas others exhibit a more indolent disease profile with a significantly better prognosis.[3] The biologic factors that contribute to the progression of this disease are poorly understood, but decreased susceptibility to apoptosis[4-6] and dysregulated proliferation undoubtedly play important roles.[7-9] This marked variability in clinical course has stimulated the search for prognostic markers that can predict patient outcome. The need for these biomarkers is particularly important now because of the introduction of more effective therapies that might be used optimally in the early phase of the disease before the problems associated with high tumor burden and drug resistance are encountered. Clinical studies have consistently shown that unmutated immunoglobulin V_H (IgV_H) genes, high zeta-chain–associated protein kinase 70 (ZAP-70) expression, high CD38 expression, and cytogenetic abnormalities (especially deletions of 11q and 17p) are all associated with a poor prognosis.[10-14] However, none of these markers are able to predict accurately the clinical course of individual patients or their likely response to therapy. This limits their use in clinical management, particularly in deciding if and when to treat. Therefore, the search for better markers is both timely and relevant.

Nuclear factor kappa B (NF-κB) proteins have long been known to be key regulators of cell survival, cell growth, immune response, and inflammation, and have been shown to be elevated in CLL cells.[15-17] This family of transcription factors transactivates more than 200 genes,[18] including a panel of antiapoptotic gene products, such as survivin, Bcl-2, and FADD-like IL-1β converting enzyme inhibitory protein, all of which have been implicated in the inhibition of cell death in CLL.[19-21] We recently showed that the NF-κB subunit Rel A is associated with in vitro survival and clinical disease progression in CLL.[15] These findings led us to hypothesize that Rel A might be a prognostic marker in this disease. We therefore analyzed Rel A DNA binding in 131 patients and correlated the results with the established markers of prognosis and clinical outcome.

PATIENTS AND METHODS

Patients

Peripheral-blood samples from 131 CLL patients were obtained after written informed consent had been received. Thirty-two (24.4%) of 131 were diagnostic samples and 112 (85.5%) of 131 patients were untreated at the start of the study. CLL was defined by clinical criteria as well as cellular morphology and the coexpression of CD19 and CD5 in lymphocytes simultaneously displaying restriction of light chain rearrangement. Comprehensive clinical information including treatment histories was available for all patients either through their case notes or direct interviews with patients. None of the previously treated patients had received chemotherapy within 3 months before sample collection. Staging was on the basis of the Binet classification system[22] and the clinical characteristics of the CLL patient cohort are summarized in Table 1.

Table 1. Patient Demographics and Clinical Characteristics of the 131-Patient CLL Cohort

Factor	No. of Patients	%
Age, years		
Median	67	
Range	36-85	
Stage at diagnosis		
A	100	77
B	16	12
C	15	11
Previous treatment		
Treated	52	40
Untreated	79	60
CD38		
< 20%	74	56
≥ 20%	57	44
Genetics		
$11q^-/17p^-$	9	7
N/O	87	66
Not determined	35	27
IgV_H gene status		
< 98%	95	73
≥ 98%	36	27
ZAP-70		
< 20%	73	56
≥ 20%	58	44

NOTE. $11q^-$ and $17p^-$ indicates any fluorescent in situ hybridization (FISH) or karyotypic abnormality of 11q or 17p. N indicates normal (no abnormality detected by FISH). O indicates other cytogenetic abnormality, excluding $11q^-$ or $17p^-$.

Abbreviations: CLL, chronic lymphocytic leukemia; IgV_H, immunoglobulin V_H; ZAP-70, zeta-chain–associated protein kinase 70.

ZAP-70 and CD38 Expression

Cytoplasmic ZAP-70 expression was determined by using a modification of a previously published flow cytometry method.[15,23] After appropriate lymphocyte gating, cytoplasmic ZAP-70 expression was determined in $CD19^+$ CLL cells by gating on the $CD3^+$ cells. Patients were considered to be ZAP-70 positive when ≥ 20% of the $CD19^+$ CLL cells had equal or greater expression of ZAP-70 than the gated $CD3^+$ cells. Cell surface expression of CD38 was examined by flow cytometry using a standard three-color flow cytometry approach of CD5-fluorescein isothiocyanate (DAKO, Copenhagen, Denmark), CD38-PE (CALTAG, Burlingame, CA), and CD19-APC (CALTAG). The cutoff point for CD38 positivity in CLL cells was ≥ 20%.

IgV_H Mutation Status

IgV_H gene mutational status was determined for all patients using the method described previously.[24] The resulting polymerase chain reaction products were sequenced and were considered unmutated if they showed 98% or greater homology with the closest germline sequence.

Subcellular Fractionation

All of the nuclear extracts were prepared in an identical fashion from freshly isolated CLL samples, and the extraction method used showed remarkable interpatient consistency in terms of the amount of nuclear and cytosolic proteins generated from a fixed number of CLL lymphocytes.[25] Details of the methods used have been described previously.[15] Protein concentrations for each nuclear extract were determined by Nanodrop absorbance (NanoDrop Technologies, Wilmington, DE).

Rel A DNA Binding Detection by Enzyme-Linked Immunosorbent Assay

Nuclear extracts from CLL patients were assayed for Rel A activity with a TransAM NF-κB transcription factor assay kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA). The principle behind the assay involves the capture of NF-κB by an oligonucleotide containing an NF-κB consensus site, which is immobilized in each well of a 96-well plate. The Rel A subunit was subsequently detected using a Rel A–specific antibody. The optical density reading at 450 nm (OD_{450}) was read on a microtiter plate reader (Bio-Rad, Hercules, CA). OD_{450} values were converted into nanograms per microgram of nuclear extract for each sample tested from a standard curve constructed using known quantities of recombinant Rel A NF-κB. In the range of Rel A proteins we were measuring, there was a linear relationship between Rel A protein amount and OD_{450} ($r^2 = 0.99$). All the Rel A measurements were done using the same batch of enzyme-linked immunosorbent assay (ELISA) kits and the same batch of recombinant Rel A protein was used for the purpose of generating standard curves. Intra- and interassay variability was assessed using standardized nuclear extracts derived from a Jurkat cell line. In addition, samples from the same patients were tested on different plates on different days. In every instance, there was less than 10% variability between results, suggesting that the method is reliable and reproducible.

Statistical Analysis

To assess the relationship between Rel A and known prognostic factors (CD38, ZAP-70, IgV_H mutation status, treatment status, and Binet stage), Rel A was examined as a continuous variable within each categoric group. To examine the relationship between Rel A and clinical outcome, the cohort was divided according to the median expression of Rel A. Overall survival and time to first treatment (TTFT) were calculated from date of diagnosis. Time to subsequent treatment (TTST) and survival during the course of the study were calculated from the date that Rel A was measured. Survival curves were constructed using the method of Kaplan and Meier, and the log-rank test was used to assess any differences between patient and tumor characteristics. Cox regression analysis determined important independent prognostic factors for TTFT and overall survival. Statistical analysis was carried out using Prism 3.0 (Graphpad Software Inc, La Jolla, CA) and SAS statistical software (SAS Institute Inc, Cary, NC).

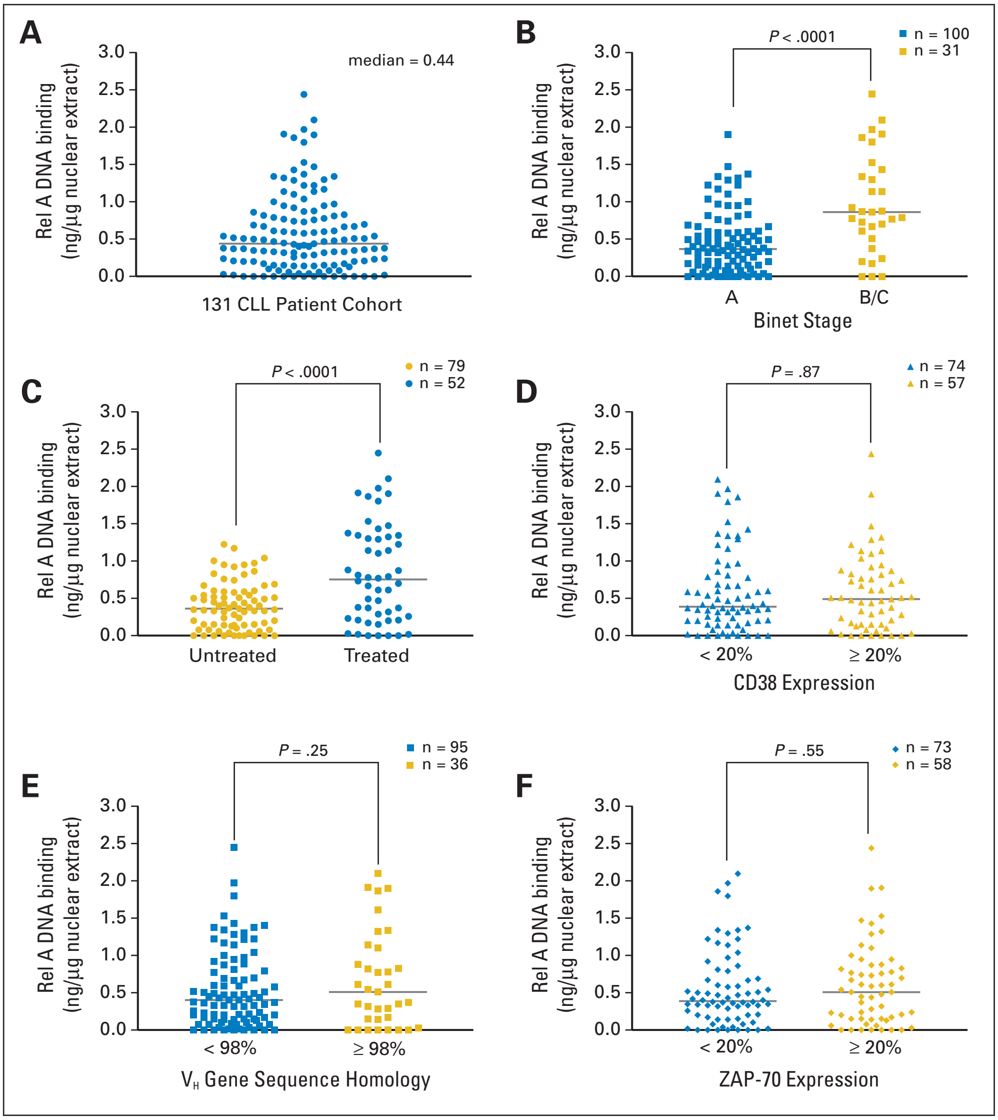

Fig 1. Correlation of Rel A and other prognostic factors. (A) Samples from all 131 patients were analyzed for Rel A DNA binding using an enzyme-linked immunosorbent assay (ELISA)–based assay. Rel A was then compared in categoric subsets of the chronic lymphocytic leukemia (CLL) cohort on the basis of (B) Binet stage, (C) the requirement for treatment, (D) CD38 expression, (E) immunoglobulin V_H (IgV_H) gene mutation status, and (F) zeta-chain–associated protein kinase 70 (ZAP-70) expression. Elevated Rel A was strongly associated with advanced Binet stage and the need for treatment, but did not correlate with CD38 expression, IgV_H gene mutation status, or ZAP-70 expression.

RESULTS

Rel A DNA Binding Is Independent of IgV_H Mutation Status, CD38 Expression, and ZAP-70 Expression

We measured Rel A in nuclear extracts derived from 131 serially collected CLL patient samples using a quantitative DNA-binding ELISA-based method. Data from all 131 patients is shown in Figure 1A; Rel A DNA binding ranged from undetectable to 2.44 ng/μg of nuclear extract, with a median value of 0.44 ng/μg (95% CI, 0.48 to 0.66). Rel A was markedly elevated in patients with advanced disease ($P < .0001$; Fig 1B) and those who had previously received treatment ($P < .0001$; Fig 1C) but was not associated with CD38 expression ($P = .87$; Fig 1D), IgV_H mutation status ($P = .25$; Fig 1E), or ZAP-70 expression ($P = .55$; Fig 1F).

Association Between Rel A DNA Binding and Time to Initial Therapy

All patients who required therapy were treated in accordance with the published guidelines.[26] Of the 131 patients analyzed, 19 (14.5%) had previously received treatment and an additional 33 (25.2%) required therapy during the course of the study. We first examined the relationship between Rel A DNA binding and the time from diagnosis to first treatment using subsets defined by the median Rel A value. The median TTFT in the low Rel A subset was 13.6 years *v* 6.8 years in the high Rel A subset ($P = .01$; Fig 2A). Because Rel A DNA binding was independent of CD38, IgV_H mutation status, and ZAP-70, we examined the ability of these established prognostic parameters to define TTFT in our cohort (Table 2). In keeping with previous reports, unmutated IgV_H genes, ≥ 20% CD38 expression, and ≥ 20% ZAP-70 expression were all associated with significantly shorter TTFT ($P = .0005$, $P = .04$, and $P = .05$, respectively), indicating that our study was carried out on a representative CLL cohort. In multivariate analysis, the inclusion of Rel A in the model showed independent prognostic significance for TTFT ($P = .01$) and its inclusion diminished the prognostic value of CD38 ($P = .25$), IgV_H gene mutation status ($P = .05$), and ZAP-70 ($P = .28$).

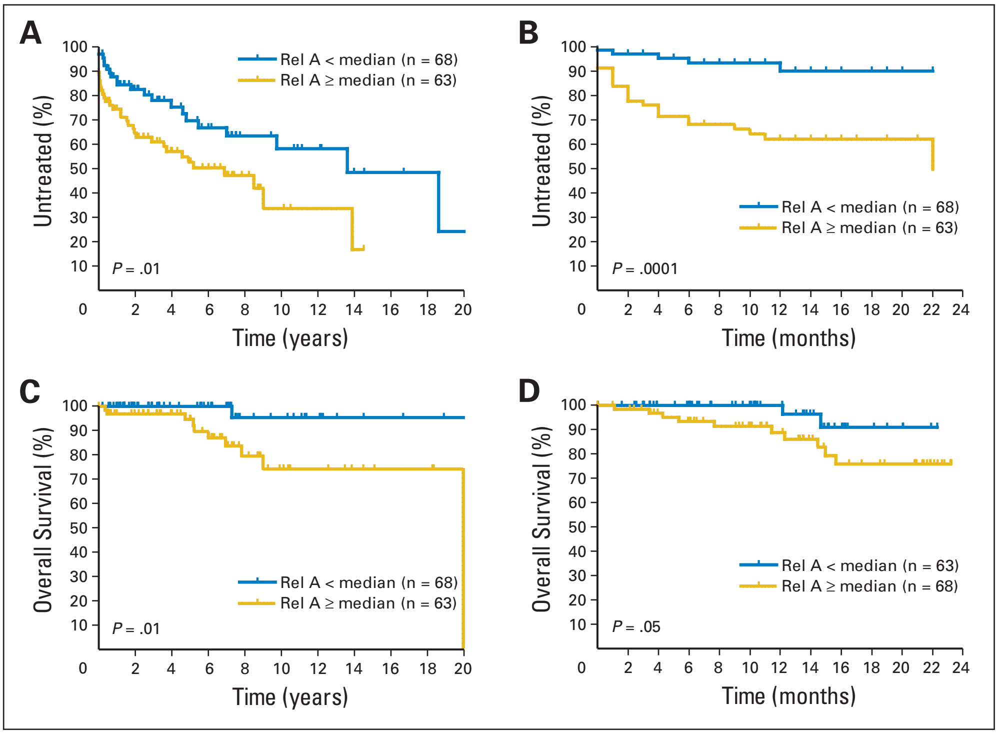

Fig 2. Rel A as a prognostic marker in our chronic lymphocytic leukemia (CLL) cohort. Time to first treatment was assessed from the date of diagnosis. (A) Rel A DNA binding above the median value was able to define a population at increased risk of requiring treatment in unit time. Time to subsequent treatment was measured from the date of entry into the study to the time of next treatment intervention. (B) Rel A DNA binding above the median value was able to define a population at greater risk of requiring another cycle of treatment. Comparison of Kaplan and Meier curves for survival demonstrated that (C) Rel A was prognostic for survival from date of diagnosis, and (D) was also predictive of survival from date of entry into the study.

Rel A Is a Predictor of Time to Subsequent Treatment

We wanted to assess more accurately the prognostic value of measuring Rel A in our cohort by controlling for previous treatment effects and to acknowledge that not all of the samples analyzed for Rel A were taken at diagnosis. Therefore we assessed TTST using the date of entry onto the study as a censor date for each patient sample. Figure 2B shows the predictive value of Rel A in our CLL patient cohort. Patients with high Rel A DNA binding showed a median TTST of 22 months. In contrast, the median TTST of the low Rel A subset was not reached, as only five of 68 patients with low Rel A DNA binding required treatment during the 24-month follow-up period ($P = .0001$). We also evaluated the ability of IgV_H mutation, CD38, and ZAP-70 to predict TTST during the study period (Table 2). Only IgV_H mutation status had prognostic value in this context ($P = .04$); CD38 ($P = .17$) and ZAP-70 ($P = .13$) were not predictive.

Rel A Is a Predictor of Overall Survival

We assessed the capacity of Rel A to predict patient survival from date of diagnosis. In addition, we assessed survival during the course of the 24-month study period using date of entry onto the study as the censor point. Rel A DNA binding was predictive of survival from date of diagnosis ($P = .015$; Fig 2C). Similarly, IgV_H mutation status, CD38, and ZAP-70 were all predictive of survival from date of diagnosis (Table 2). In multivariate analysis, the inclusion of Rel A in the model showed independent prognostic significance for overall survival ($P = .04$), and its inclusion removed the prognostic value of CD38 ($P = .38$), IgV_H gene mutation status ($P = .22$), and ZAP-70 ($P = .14$). Furthermore, only Rel A ($P = .01$; Fig 2D) was predictive of survival during the course of the 24-month follow-up period of this study.

Rel A Is Constitutively Higher in Patients Who Require Treatment

For Rel A to be considered a useful marker of TTFT in CLL, it seemed important to demonstrate that Rel A was constitutively higher in untreated patients who went on to require therapy. We therefore compared Rel A DNA binding in samples derived from patients who never required therapy (n = 79) with those who went on to receive their first treatment after we had measured their Rel A DNA binding

Table 2. Comparison of Prognostic Factors in Terms of Time to Treatment and Overall Survival

	Time to Treatment						Survival					
	Time to First Treatment			Time to Subsequent Treatment			From Date of Diagnosis			From Date of Entry Into Study		
Factor	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Rel A (< median/≥ median)	1.9	1.15 to 3.41	.01	5.2	2.00 to 8.61	.0001	9.1	1.45 to 15.51	.01	3.9	0.97 to 9.59	.05
IgV_H gene mutation (< 98%/≥ 98%)	2.7	1.84 to 9.01	.0005	2.1	1.04 to 4.73	.04	3.9	1.66 to 40.29	.01	1.2	0.38 to 4.05	.71
CD38 expression (< 20%/≥ 20%)	1.6	1.00 to 2.80	.05	1.6	0.79 to 3.46	.17	2.8	0.96 to 9.82	.05	1.3	0.25 to 2.42	.66
ZAP-70 expression (< 20%/≥ 20%)	1.7	1.00 to 2.98	.04	1.7	0.84 to 3.66	.13	3.1	1.01 to 9.17	.05	1.6	0.53 to 5.13	.39

Abbreviations: HR, hazard ratio; IgV_H, immunoglobulin V_H; ZAP-70, zeta-chain–associated protein kinase 70.

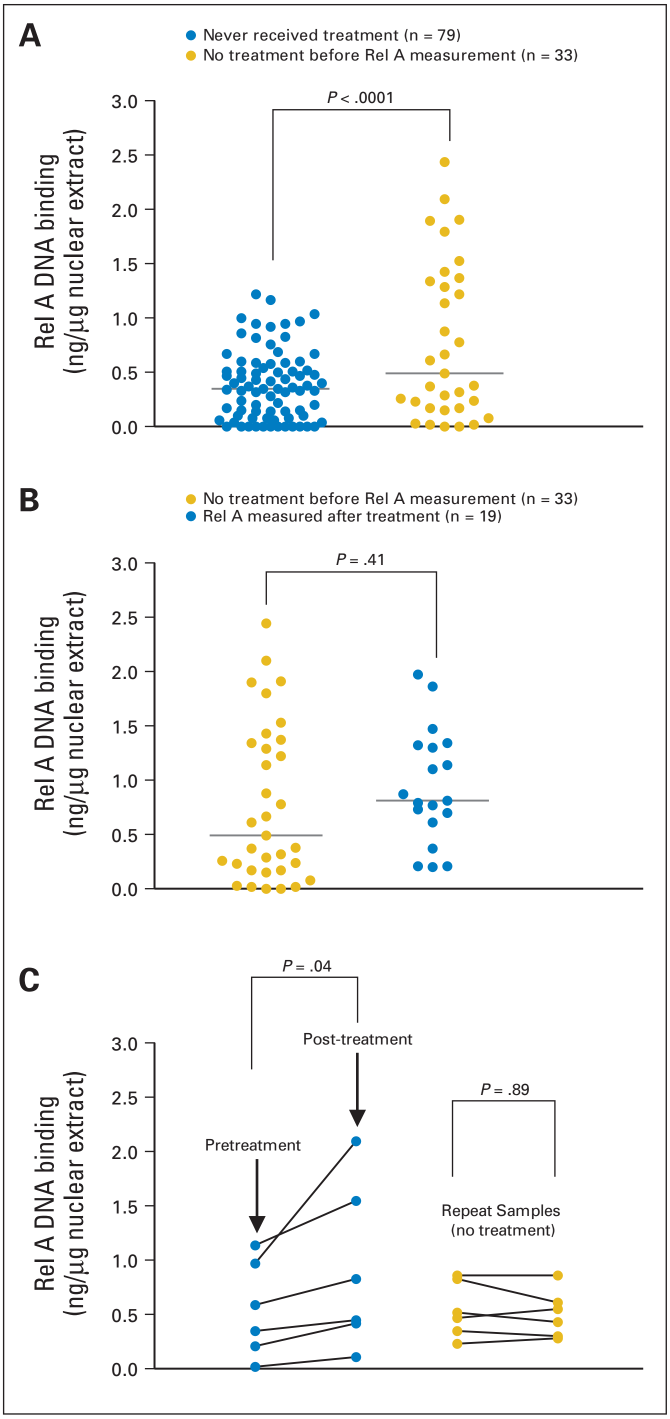

Fig 3. The effect of treatment on Rel A DNA binding. (A) Rel A DNA binding was significantly higher in samples derived from previously treated patients ($P < .0001$). (B) However, there was no statistical difference in Rel A DNA binding in samples analyzed before treatment and those in whom Rel A was measured after treatment ($P = .41$). (C) Serial intrapatient analysis of Rel A in samples derived from patients who received treatment during the course of the study and those who did not revealed that there was a significant increase in Rel A DNA binding after therapy ($P = .04$). In contrast, samples from patients who did not require treatment remained stable ($P = .89$).

(n = 33). Figure 3A shows that patients who require therapy have significantly higher Rel A DNA binding than those patients who do not ($P < .0001$).

Is Rel A Modulated by Therapy?

Studies of other diseases have shown that Rel A DNA binding can increase in response to chemotherapeutic drugs.[27-29] We examined this question in two ways. First, we compared Rel A DNA binding in samples derived from patients who received therapy after we had measured their Rel A with those who received therapy before we measured their Rel A (Fig 3B). Although there was no statistical difference in Rel A DNA binding between these two groups ($P = .41$), the median Rel A DNA binding was higher in the previously treated subset (median Rel A, 0.81 *v* 0.49 ng/µg). Second, we evaluated intrapatient Rel A DNA binding in a longitudinal manner in patients before and after treatment and in selected patients who did not require therapy. Figure 3C shows a clear increase in Rel A DNA binding after treatment ($P = .04$). In contrast, serial samples from patients not requiring therapy showed no significant change in Rel A DNA binding ($P = .89$). None of the samples tested were derived from patients within 3 months of completion of a cycle of therapy to avoid the possibility of a transient modulation in Rel A DNA binding induced by chemotherapeutic drugs. The median interval between measurements was similar for the treated and untreated patient groups (15 *v* 13 months, respectively).

DISCUSSION

A number of previous studies have shown that NF-κB is constitutively activated in CLL patients.[15-17] Here we used an ELISA-based assay to demonstrate marked variation in Rel A DNA binding within a cohort of 131 unselected CLL patients. The Rel A DNA binding assay that we used was both reliable and reproducible with less than 10% variation in biologic replicates performed on different plates on different days. High Rel A DNA binding was strongly associated with advanced stage of disease, but remarkably, was not associated with IgV_H gene mutation status, CD38 expression, or ZAP-70 expression, indicating that Rel A could contribute independent prognostic information in CLL. We therefore assessed the ability of Rel A to predict for TTFT, TTST, and overall survival in our patient cohort.

Elevated Rel A DNA binding was predictive of significantly shorter TTFT (hazard ratio [HR], 1.9; $P = .01$). Given that NF-κB has been implicated in the development of drug resistance in other tumor settings,[30,31] we assessed whether Rel A could predict for the relative effectiveness of therapy in CLL patients. To do this, we used TTST from the date of entry onto the study as a measure of the depth of remission. Rel A was more predictive of TTST than any of the other prognostic markers assessed (HR, 5.2; $P = .0001$), indicating that it is excellent biomarker of response in CLL patients. Although this result is encouraging, it will need to be tested more thoroughly in a prospective clinical trial in which all of the patients receive uniform therapy. Most strikingly, Rel A was the best predictor of overall survival from date of diagnosis in our study (HR, 9.1; $P = .01$), adding to our hypothesis that Rel A is a key regulator of CLL survival both in vitro and in vivo.[15] In keeping with this notion, Rel A was the only parameter tested that showed a significant difference in survival during the 24-month follow-up period of this study (HR, 3.9; $P = .05$). Furthermore, the inclusion of Rel A in multivariate modeling showed the independent importance of Rel A in determining overall survival in our cohort (HR, 8.0; $P = .05$); CD38 expression, IgV_H mutation status, and ZAP-70 expression all failed to influence survival in the presence of Rel A.

NF-κB can be activated in response to chemotherapy and can promote drug resistance through the induction of antiapoptotic proteins like Bcl-2, survivin, and FADD-like IL-1β converting enzyme inhibitory protein, as well as the activation of the *AKT* survival pathway.[19-21,32] We found that Rel A DNA binding was significantly higher in samples derived from previously treated patients ($P < .0001$) and clearly increased in serial samples tested pre- and post-therapy. However, constitutive Rel A was significantly higher in patients who went on to require treatment when compared with those who did not. Furthermore, high Rel A was also associated with a shorter TTST in our cohort. Taken together, these data indicate that Rel A is constitutively elevated in patients with more aggressive disease but is also induced by conventional chemotherapy, which in turn seems to contribute to the depth of response to subsequent treatment cycles.

In conclusion, this study identifies Rel A as a superior prognostic marker for survival in CLL and crucially demonstrates that Rel A has the potential to predict the duration of response to therapy.[33] Although there are undoubtedly challenges involved in the routine measurement of Rel A DNA binding in clinical practice, our work shows that it is possible to generate robust and reliable results that have clinical importance using a widely available measurement platform. In addition, our findings strongly support the search for more accessible surrogate markers of NF-κB activation in this disease. The fact that Rel A DNA binding is independent of IgV_H mutation status, CD38 expression, and ZAP-70 expression offers the possibility that the prognostic information derived from some or all of these factors could be combined to provide even more accurate prediction of clinical outcome for CLL patients in the future.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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