Human Cancer Biology

Aggressive Chronic Lymphocytic Leukemia with Elevated Genomic Complexity Is Associated with Multiple Gene Defects in the Response to DNA Double-Strand Breaks

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Abstract

Purpose: Genomic complexity is present in approximately 15% to 30% of all chronic lymphocytic leukemia (CLL) and has emerged as a strong independent predictor of rapid disease progression and short remission duration in CLL. We conducted this study to advance our understanding of the causes of genomic complexity in CLL.

Experimental Design: We have obtained quantitative measurements of radiation-induced apoptosis and radiation-induced ATM autophosphorylation in purified CLL cells from 158 and 140 patients, respectively, and have used multivariate analysis to identify independent contributions of various biological variables on genomic complexity in CLL.

Results: Here, we identify a strong independent effect of radiation resistance on elevated genomic complexity in CLL and describe radiation resistance as a predictor for shortened CLL survival. Furthermore, using multivariate analysis, we identify del17/p53 aberrations, del11q, del13q14 type II (invariably resulting in Rb loss), and CD38 expression as independent predictors of genomic complexity in CLL, with aberrant p53 as a predictor of ~50% of genomic complexity in CLL. Focusing on del11q, we determined that normalized ATM activity was a modest predictor of genomic complexity but was not independent of del11q. Through single nucleotide polymorphism array-based fine mapping of del11q, we identified frequent monoallelic loss of Mre11 and H2AFX in addition to ATM, indicative of compound del11q–resident gene defects in the DNA double-strand break response.

Conclusions: Our quantitative analysis links multiple molecular defects, including for the first time del11q and large 13q14 deletions (type II), to elevated genomic complexity in CLL, thereby suggesting mechanisms for the observed clinical aggressiveness of CLL in patients with unstable genomes. Clin Cancer Res; 16(3); 835–47. ©2010 AACR.

Genomic aberrations substantially influence the biology and clinical outcome of patients with chronic lymphocytic leukemia (CLL; refs. 1–3). The presence of specific chromosomal deletions, in particular del17p and del11q, allows for the identification of a subset of patients with shortened overall survival. Furthermore, CLL patients with chromosomal translocations, complex aberrant karyotypes, or elevated genomic complexity, as measured through single nucleotide polymorphism (SNP) arrays, have recently been shown to have aggressive CLL that is characterized by rapid disease progression and short remission duration (4–9). An ad hoc summary analysis of these published reports suggests that CLL cases with these latter genomic complexity characteristics are substantially more common than CLL with del17p or p53 mutations and that the overlap between these two groups of CLL is partial, thus implying the existence of additional unidentified factors that cause genomic complexity in CLL.

Subchromosomal deletions, which are the quantitatively dominant genomic aberration in CLL, must have involved DNA double-strand breaks as intermediates. Given that the cellular response to DNA double-strand breaks comprises cell cycle arrest, repair pathway activation, or, alternatively, apoptotic cell death, it seems rational to assume that defects in DNA double-strand break repair/response pathways are causally linked to subchromosomal genomic complexity in CLL. The two most prominent examples of genes, mutated or defective in subsets of CLL, which are part of the DNA double-strand break repair/response pathways are p53 and ATM, and both genes have been linked to more aggressive CLL (1, 10–15). Nonetheless, mutations or aberrations leading to the reduced function of either gene are not routinely comprehensively measured in CLL, and further,
Translational Relevance

Chronic lymphocytic leukemia (CLL) has a varied clinical course. The identification of genomic markers that predict for CLL survival has motivated interests in genomic predictors of outcome. Genomic complexity that exists in 15% to 30% of nontreated CLL patients is a strong independent negative prognostic marker in CLL. In this article, we have comprehensively analyzed genomic complexity determinants, and results from this work further strengthen the concept of impaired DNA double-strand break response/repair pathways in CLL and further identify genomic complexity as the product of multiple gene alterations in CLL. These data further motivate the development of diagnostic and therapeutic approaches to incorporate this knowledge into CLL clinical care.

the relationship of aberrations in either gene to CLL with complex genomes has not been systematically investigated in large prospectively analyzed CLL cohorts.

One of the attractive features of genomic complexity assessments as a biomarker for aggressive CLL is the fact that establishing a link between the ineffectiveness of commonly used genotoxic drugs in CLL, an impaired DNA double-strand break apoptotic response, and elevated genomic complexity would provide a firm rationale to target CLL with elevated genomic complexity with alternative or nongenotoxic therapies. As many of the most commonly used drugs in CLL therapy, including purine analogues and cyclophosphamide, are genotoxic and, in the case of fludarabine, have been shown to induce a p53-dependent transcriptional response in CLL cells, assessments of elevated genomic complexity could lead to the development of risk-adapted therapies akin to therapies developed for patients with del17p (16–18).

In addition to genomic aberrations, various biomarkers including ZAP-70 expression, IgVH status, CD38 expression, and others have been identified in CLL that can dichotomize CLL patient populations into lower and higher risk groups (19–30). However, little information is available about a possible link between these various biomarkers and genomic complexity in CLL.

In this study using multivariate analysis, we have comprehensively analyzed the effect of various measurable biomarkers on genomic complexity in CLL. Furthermore, we discovered an impaired cellular apoptotic response to radiation as an independent strong predicting factor for complexity in CLL, thus providing direct experimental evidence for an involvement of an impaired cellular response to DNA damage in the acquisition of genomic lesions. From these data, we conclude that an impaired DNA double-strand break response and multiple genomic deletions, including del17p, del11q, and del13q14 type II (Rb loss), are independent strong predictors of genomic complexity in CLL. Combined, these data suggest the existence of multiple independent gene defects in CLL that confer genomic instability. Among these defects, we identify a strong independent effect of aberrant p53 function on genomic complexity in CLL and, surprisingly, only a modest effect of ATM hypofunction. We identify multiple genes in the response pathway following DNA double-strand breaks (ATM, Mre11, and H2AFX) to be codeleted through del11q, thus suggesting that the genomic complexity associated with del11q may be caused by compound gene defects (31). Finally, we report for the first time an independent strong effect of del13q14 type II deletions on genomic complexity (32) and have identified reduced Rb expression associated with these larger 13q14 subtypes.

Materials and Methods

Patients. This study is based on CLL patient samples that were as described in refs. (6, 12). The trial was approved by the University of Michigan Institutional Review Board (IRBMED #2004-0962) and written informed consent was obtained from all patients before enrollment. Clinical outcome analysis followed previously published definitions (6).

Measurements of radiation-induced apoptosis in CLL. Cryopreserved CLL cells were thawed, washed, and depleted of CD3- and CD14-positive cells using negative selection over Miltenyi Biotec LS columns. Tumor cells thus obtained were purified and aliquoted into low-cell-binding tissue culture plates (Nunc #145385) at concentrations of 1.2 × 10⁶ per milliliter for Western blotting and 3 × 10⁶ per milliliter for apoptosis analysis by flow cytometry. Cells rested for 1 to 1.5 h before treatment with 5 Gy of ionizing radiation (Philips 250 kV X-ray-emitting source). Apoptosis measurement by flow cytometry was carried out after cells had incubated at 37°C with 5% CO₂ for 40 to 42 h postirradiation, using Annexin V and propidium iodide (PI) staining and a Becton Dickinson FACSCalibur flow cytometer. Duplicate measurements of remaining viable cells (Annexin V-negative/PI-negative) for irradiated and paired nonirradiated tumor samples were tabulated.

Detection of p53 aberrations. Sequence analysis of p53 exons 5 to 9 was available for all studied samples as previously described (12). In addition, p53 immunoblotting results from CLL cells treated with MDM2 inhibitors or solvent were available for 106 of 178 of the samples as previously described. Given that an aberrant p53 immunoblotting result was never found in the 106 tested samples in the absence of either known p53 sequence mutations, the presence of del17p, or copy-neutral loss of heterozygosity at 17p (all data available for the entire cohort), we grouped all remaining CLL (n= 72) with wild-type p53 exon 5 to 9 and the absence of either del17p or acquired copy-neutral loss of heterozygosity (aUPD) at 17p as p53 nonaberrant.

Exon resequencing of Mre11a and H2AFX. Primers to amplify and sequence all coding exons of human Mre11a and H2AFX and adjacent intronic sequences were designed.
using the primer 3 program. The sequences of the primers used are tabulated in Supplementary Table S1. Conditions for PCR amplifications were optimized using gradient temperature PCR conditions and are available upon request. PCR products were generated using Repli-g (Qiagen)-amplified DNA from highly pure sorted CD19+ cells as templates and analyzed as described in ref. (12). Mutations were confirmed in un amplification genomic DNA and paired CD3+ cell-derived DNA.

Measurements of radiation-induced ATM autophosphorylation in CLL. Cells for Western blot were harvested 20 min after irradiation treatment, washed once with 1× PBS, and lysed on ice for 20 min with lysis buffer (50 mM Tris, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, and 20 mM NaF) supplemented with fresh protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, and 1× Sigma protease and phosphatase inhibitor cocktails). Western blots were carried out for both irradiated and nonirradiated CLL-derived tumor samples for phospho-ATM (Ser1981, Rockland Immunocoehemicals), total ATM (Santa Cruz Biotech), actin (Sigma), and α-tubulin (Santa Cruz) for all patients. All Western blots included two pooled standard samples and uniform cell equivalent loading to enable quantitation across multiple blots. Western blots for phospho-Ser-1981-ATM, ATM, and α-tubulin (Santa Cruz) for all patients. All

Duplicate amplification reactions included primers/probes, Taqman 2× Universal PCR Master Mix, No AmpErase UNG, and 1 μL of cDNA in a 20-μL reaction volume. Reactions were done on an ABI 7900HT machine. Normalization of relative copy number estimates for Rh, Mre11a, or H2AFX RNA was done with the Cycle threshold (Ct) values for PGK1 as reference (Ct mean gene of interest – Ct mean PGK1).

Statistical methods. Genomic complexity was defined as having three or more losses, or having three or more losses plus gains plus uniparental disomy (UPD) – total complexity. Logistic regression was used to relate the presence of genomic complexity (a dichotomous response variable) to potential predictive factors of complexity. All factors of interest were considered in univariate analysis. ALL–fluorescence in situ hybridization (All-FISH) data categorize samples independent of degree of clonal involvement; FISH-25 data categorize samples only if ≥25% of nuclei carried the FISH finding as per routine clinical FISH reports from the Mayo Clinic. Mean positive cell fraction for FISH categories in this cohort was 68% for del17p, 59% for del11q, and approximately 75% for del13q14 type II. In multivariate analysis, all the models we considered included a core set of four variables: CD38, ZAP-70, IgVH, and del13q type II. Three additional factors were then added to each model: one of the del11q measurements (FISH-25 or All-FISH), one of the phospho-ATM measurements, and one of the following four factors: p53 aberrancy status, percent of cells alive post-irradiation, and del17p either as FISH-25 or All-FISH. The latter four factors were grouped because they are mechanistically related. Patients with multiple genomic abnormalities were counted in each category without the use of a hierarchical model. For both univariate and multivariate analysis, the relationship between genomic complexity and a given predictive factor was assessed by the odds ratio (OR) (conditional OR in multivariate analysis) and its p value. The polarity of each variable was assigned so that higher levels of coding corresponded to greater univariate risk of complexity. Thus, higher risk is associated with greater CD38 and ZAP-70 levels; unmutated IgVH lower normalized phospho-ATM levels; higher percentages of cells alive postirradiation; p53 aberrations; and the presence of del13q, del11q, and del17p deletions.

Survivor functions, time to first; time to subsequent therapy; and overall survival (TTFT, TTST, and OS) for CLL subgroups defined by the degree of radiation resistance or ATM activity (measured through p-ATM/ATM or p-ATM/actin ratios) are displayed using the method of Kaplan and Meier.

Results

Patient characteristics. Included in this analysis are data from 178 patients that were previously analyzed for genomic complexity using 50K SNP arrays (6). Data on individual biomarkers including ZAP-70 expression, IgVH status, CD38 expression, p53 mutations, CLL FISH, 50K SNP-array data, and MDM2 SNP309 allele status were available for all of the 178 cases (6). Depending on availability of sufficient cryopreserved specimens for analysis,
radiation-induced apoptosis data were available for 158 cases; normalized p-ATM data were available for 141 cases; and normalized Rb mRNA data were available for 160 cases. This core data set was supplemented with additional consecutively enrolled CLL cases specifically for del11q breakpoint mapping using 6.0 SNP array data (data not shown) and Mre11a mRNA analysis using Q-PCR (n = 220). All analysis described below is solely based on biological specimens collected at the time of study enrollment, thus minimizing bias introduced through CLL clonal evolution or biomarker instability.

Impaired radiation-induced apoptosis is a strong predictor of genomic complexity in CLL. Based on unbiased measurements of subchromosomal lesion loads in CLL using 50K SNP arrays (derived from pairwise analysis of CD19+ cells and paired buccal DNA), we had previously defined acquired genomic complexity in CLL as the sum of all subchromosomal losses (loss complexity) or the sum of subchromosomal losses, gains, and UPD (total complexity) per CLL case, respectively (6). Given that molecular defects in genes involved in DNA double-strand break response and repair pathways are likely candidates for causing genomic complexity, we used external radiation to introduce DNA double-strand breaks into CLL cells from 158 patients and measured the cellular apoptotic response to radiation approximately 40 to 44 hours after the

![Fig. 1. Measurements of the apoptotic response of CLL cells to radiation and the association with elevated genomic complexity. Highly enriched CD19+ cells from 158 CLL patients were irradiated with 5 Gy, and the viable and apoptotic/necrotic cell populations were measured for irradiated and nonirradiated samples using Annexin V-PI fluorescence-activated cell sorting staining ∼40 h postirradiation. Each dot represents the mean normalized fraction of duplicate measurements of cells alive (%alive = % alive irradiated samples/% alive paired nonirradiated samples) for individual CLL samples categorized by hierarchical FISH (all depicted genomic categories other than p53 aberrant or del17p are p53 wild-type). Colored dots, cases with genomic complexity with three or more lesions. Horizontal bars, the mean of values within each genomic category. A, loss complexity. B, total complexity.](http://clincancerres.aacrjournals.org/content/16/3/838)
radiation insult. Data are summarized in Fig. 1 with the normalized fraction of cells alive after radiation plotted against hierarchical CLL FISH data (all genomic categories other than p53 aberrant or del17p are p53 wild-type. Furthermore, all del17p cases depicted were p53 aberrant, whereas a few p53 aberrant cases either lacked del17p or carried aUPD at 17p that is not detectable through FISH).

As can be seen in Fig. 1, CLL cells from patients with either del17p or p53 aberrations (see Materials and Methods) were resistant to radiation-induced apoptosis (mean normalized fraction of cells alive postirradiation of 1.14 and 1.03, respectively), confirming the essential role that p53 serves in the apoptotic response to radiation-induced DNA double-strand breaks and providing confidence in our analytic conditions. Similar analysis for CLL cells with del11q, trisomy 12, or del13q14 type II identified intermediate radiation sensitivity (mean normalized fraction of cells alive postirradiation of 0.5, 0.54, and 0.41, respectively), and analysis for isolated del13q14 type I or normal FISH identified substantial radiation sensitivity (mean

**Fig. 2.** Ex vivo radiation resistance of CLL cells predicts for progressive and aggressive CLL (Kaplan-Meier plots). CLL cases analyzed were grouped by tertiles. A to C, n = 158 inclusive of p53 aberrations; D to F, n = 139 exclusive of p53 aberrations. A and D, TTFT estimates; B and E, TTST estimates; C and F, overall survival. High, highest radiation resistance.
normalized fraction of cells alive postirradiation of 0.26 and 0.31, respectively). Compared with the CLL cases with isolated del13q14 type I, all genomic CLL categories, except for normal FISH, displayed highly significant radiation resistance. Interestingly, in addition to the CLL with del17p or p53 aberrations, each of the other five FISH-based CLL categories encompassed multiple cases that showed profound radiation resistance even in the absence of p53 aberrations. Thus, significant defects in the apoptotic response to DNA double-strand breaks other than p53 are present in a substantial fraction of CLL.

Next, we highlighted the subset of CLL cases with a genomic complexity of three or more lesions within the radiation resistance/sensitivity plots (Fig. 1): most CLL cases with elevated complexity and del17p/p53 aberrations or del13q14 type II were characterized by substantial or complete radiation resistance. Sporadic CLL cases with elevated genomic complexity in all other FISH categories (including del11q) showed either partial radiation resistance or relative radiation sensitivity. Furthermore, CLL cases with trisomy 12, isolated del13q14 type I, or normal FISH were only rarely associated with elevated complexity. Finally, CLL cases with trisomy 12, despite being associated with substantial resistance to radiation-induced apoptosis, did not display genomic complexity, thus indicating that defects in radiation-induced apoptosis are heterogeneous and that some defects are not sufficient to cause unstable genomes in CLL.

Radiation resistance identifies aggressive CLL. Next, we analyzed the prognostic effect of ex vivo radiation resistance on clinical outcome variables in CLL within the above-mentioned set of 158 CLL cases. Furthermore, we separately analyzed the cases within this group that had wild-type p53 (n = 139) as defined above.

As can be seen in Fig. 2A to F, ex vivo radiation resistance was an excellent predictor of aggressive CLL with shortened TTFT, TTST, and OS when comparing three equal sized groups of CLL cases characterized by the lowest, intermediate, or highest radiation resistance. Importantly, this remained true for the cohort that excluded p53 aberrations (Fig. 2D-F).

CLL cases with elevated genomic complexity are identified across the spectrum of FISH-defined genomic subtypes. We analyzed the occurrence of elevated genomic complexity with three or more lesions in all FISH25-defined genomic subgroups in our cohort of 178 CLL. As can be seen in Table 1 and Fig. 3A and B, various genomic CLL subgroups (del17p/p53 aberrations, del11q, del13q14 type II, and del6q) were characterized by a high proportion of cases with elevated complexity, whereas conversely, CLL with trisomy 12, del13q14 type I, or normal FISH were infrequently complex.

Next, we analyzed the sensitivity and specificity of a FISH result with two or more lesions (e.g., del17p/del13q14) for genomic complexity at either two or more, three or more lesions for both complexity based on losses or total complexity (FISH results with three or more lesions had an incidence of only 2% in this cohort). As summarized in Supplementary Table S2, FISH with two or more lesions was not sensitive (approximately 45-50%) but quite specific (~90%) for genomic complexity.

Identification of predictors of genomic complexity in CLL using univariate analysis. Next, we wished to determine the importance of various biomarkers, including the apoptotic response of CLL cells to radiation, as predictors of genomic complexity in CLL. Genomic complexity was dichotomized into less than three or three or more lesions, a cutoff that had previously allowed for the identification of a subset of CLL with aggressive disease. For the following univariate and multivariate analysis, none of the variables were subjected to a hierarchical model.

In univariate analysis, multiple factors emerged as significant predictors of genomic complexity in CLL (see Supplementary Table S3). For instance, in the analysis using complexity at three or more losses, p53 aberrations (OR = 17.75; P < 0.001), del17p (OR = 23.62; P < 0.0001), del11q (OR = 7.42; P < 0.0001), del6q (OR = 12.33; P = 0.005), the fraction of cells alive postirradiation (OR = 8.62 for any 50% increment in cells alive (for instance 75% alive versus 25% alive), P < 0.0001], IgVH unmutated (OR = 4.38; P = 0.002), CD38 (OR = 3.44; P = 0.005), del13q14 type II (OR = 2.92; P = 0.018), ZAP-70 (OR = 3.92; P = 0.003), and lower Rb mRNA levels (coded quantitatively with lower Rb mRNA levels reflected in higher ∆Ct Rb-PGK1 levels and higher ORs for complexity) emerged as predictive factors. If only FISH25 results were considered, the corresponding results were del17-FISH25 (OR = 20.28; P < 0.0001), del11q-FISH25 (OR = 11.6; P < 0.0001), and del6q FISH-25 (OR = 12.33; P = 0.005). The MDM2 SNP 309 allele status (TT versus either T/G or GG) and del13q14 type I did not emerge as significant risk factors (33). The presence of trisomy 12 or normal FISH was negatively associated with genomic complexity.

ATM activity is a modestly strong predictor of genomic complexity in CLL in univariate analysis. Given the strong effect of del11q on genomic complexity in CLL and the

**Table 1. Absolute number of CLL cases with specific genomic abnormalities and elevated genomic complexity out of a total of 178 patients (all nonhierarchical FISH 25 based)**

<table>
<thead>
<tr>
<th>Genomic category</th>
<th>No. of CLL with loss complexity*</th>
<th>No. of CLL with total complexity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 aberrant</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>del 17p</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>del 11q</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>trisomy 12</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>del 13q type I</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>del 13q type II</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>del 6q</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Normal FISH</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>

*Cohort size = 178 cases.
reported association of del11q with ATM mutations/dysfunction, we proceeded with measurements of normalized ATM activity in all studied CLL cases for which sufficient cryopreserved cells were available (n = 141). ATM autophosphorylation on Ser-1981 was measured 20 min after 5 Gy of radiation and was normalized on each immunoblot to internal standards and subsequently to normalized levels of total ATM or actin. Normalization to both total ATM and actin allowed further separation of ATM hypoaactivity due to low ATM expression (p-ATM/ATM preserved, p-ATM/actin low) or dysfunctional ATM (p-ATM/ATM and p-ATM/actin both low). Representative immunoblots are shown in Supplementary Fig. S1. Mean and median p-ATM/ATM and p-ATM/actin ratios were 0.93:0.92 and 1.13:1.08, respectively, with a range of measurements of 0.0 to 2.41 and 0.0 to 2.95, respectively. Of these measurements, 117:141 (83%) and 116:141 (82%) of p-ATM/ATM and p-ATM/actin ratios fell between 0.5 and 2, and 24:141 (17%) and 25:141 (18%) of p-ATM/ATM and p-ATM/actin ratios were <0.5. Furthermore, inspection of displays of p-ATM/ATM and p-ATM/actin ratios as in Fig. 4 suggested ATM function in CLL to be a continuous variable without overt clustering, except for a small subset of CLL cases showing profound loss of ATM activity and/or protein [only 8:141 (6%) and 14:141 (10%) of p-ATM/ATM and p-ATM/actin ratios were <0.2]. Finally, of the 10 CLL cases with del11q and available measurements, 5 had p-ATM/actin ratios of <0.1, providing additional confidence in our assay conditions.

Next, we determined the predictive effect of p-ATM/ATM and p-ATM/actin ratios on genomic complexity in CLL using these ratios as a continuous variable. Using univariate analysis, low p-ATM/ATM and p-ATM/actin ratios showed a modest predictive effect on genomic complexity (p-ATM/ATM OR = 2.87; \( P = 0.057 \); and p-ATM/actin OR = 2.69; \( P = 0.019 \) for three or more versus less than three losses; and p-ATM/ATM OR = 2.92; \( P = 0.036 \) and p-ATM/actin OR = 2.10; \( P = 0.043 \) for three or more versus less than three total lesions) for any absolute decrease in the ratios of 1 (for instance, p-ATM/ATM ratios of 1.6 and 0.6, respectively).

ATM activity is not a predictor of outcome in CLL. We analyzed the effect of p-ATM/ATM and p-ATM/actin ratios in three equal-sized groups of CLL (lowest, intermediate, and highest ATM activity) on the outcome variables TTTI, TTST, and OS in the cohort of cases with available

![Fig. 3. Elevated genomic complexity and relationship to CLL FISH categories. A and B, frequency of elevated genomic complexity based on losses or total complexity in various genomic CLL categories.](https://example.com/image.png)
measurements. As depicted in Supplementary Fig. S2A to F, ATM activity was not a significant prognostic variable in CLL.

**Del17p/p53 aberrations, del11q, del13q14 type II, and CD38 expression are independent predictors of genomic complexity in CLL (multivariate analysis).** We proceeded with multivariate analysis to identify dependent and independent predictors of genomic complexity in CLL and included the following variables in parallel Cox proportional hazard models: CD38, ZAP-70, IgVH status, del13q14 type II, and del11q; alternatively, either del17p, p53 aberrations, or the fractions of cells alive postirradiation as the latter three variables are directly linked mechanistically through p53. Data are summarized in Table 2, grouped by results for loss complexity (A) and total complexity (B).

From these models, we identified p53 aberrations/del17p, del11q, and del13q14 type II as strong independent predictors of elevated genomic complexity in CLL. In models including the variable % cells alive postirradiation, del11q lost independence, consistent with the notion that the del11q effect is linked to a defective response to DNA double-strand breaks. Furthermore, CD38 expression in >30% of CLL cells was an independent complexity predictor, whereas ZAP-70 expression and IgVH status were not.

Next, we added the variables p-ATM/ATM or p-ATM/Actin into the models. Data are summarized in Supplementary Table S4 and S5. From these models, it became clear that ATM activity was not independent of del11q, thus providing novel evidence for del11q-associated genes other than or in addition to ATM that confer genomic complexity on CLL cells.

**Del11q frequently results in monoallelic deletions of Mre11 or H2AFX in addition to invariate monoallelic ATM deletion.** Most CLL cases with del11q had elevated genomic complexity but this was not strictly dependent on absent ATM activity. For instance, of the 10 CLL cases with del11q and available measurement of ATM activity and wild-type p53, 3 of 5 cases with complete loss of ATM activity had elevated genomic complexity, as opposed to 3 of 5 cases with essentially normal ATM activity (see Table 3 and Fig. 5).

We therefore reviewed the anatomy of del11q as defined through SNP array profiling of 232 CLL cases for additional complexity-causing candidate genes. Overall, SNP array analysis detected 22 of 232 cases with del11q that spanned ATM. Of these, 22 of 22 (100%) resulted in the loss of one copy of ATM, 11 of 22 (50%) included Mre11, 4 of 22 (18%) included H2AFX, and 14 of 22 (64%) cases included either Mre11 or H2AFX or both together with ATM loss (see Table 3 and Fig. 5). Therefore, multiple genes with important roles in the response pathways activated by DNA double-strand breaks are monoallelically codeleted through del11q in the majority of CLL cases.

Next, we measured normalized Mre11a expression in RNA from fluorescence-activated cell sorting–sorted CD19+ cells (n = 220 CLL cases) and grouped resulting normalized
values according to del11q status and Mre11a status. As can be seen in Supplementary Fig. S3A, Mre11a mRNA levels were substantially and significantly lower in CLL with del11q that was inclusive of Mre11a than in CLL with del11q exclusive of Mre11a or all other non-del11q CLL cases.

To obtain additional evidence in support of a contributing role of Mre11a in del11q biology and genomic complexity in CLL, we sequenced all coding exons of Mre11a in all CLL that carried del11q and monoallelic Mre11a loss (n = 11). We identified two CLL cases with Mre11a nucleotide changes as a consequence of loss of heterozygosity at del11q.

Table 2. Multivariate analysis results of the OR of various markers/biomarkers as predictors of genomic complexity

<table>
<thead>
<tr>
<th>Biomarker/variable, n = 172</th>
<th>OR</th>
<th>P</th>
<th>Biomarker/variable, n = 172</th>
<th>OR</th>
<th>P</th>
<th>Biomarker/variable, n = 156</th>
<th>OR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Loss complexity (three or more lesions)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>P53 status</td>
<td>21.64</td>
<td>&lt;0.01</td>
<td>Del17p</td>
<td>26.63</td>
<td>&lt;0.01</td>
<td>Cells % alive postirradiation</td>
<td>7.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Del11q</td>
<td>7.27</td>
<td>0.01</td>
<td>Del11q</td>
<td>5.66</td>
<td>0.01</td>
<td>Del11q</td>
<td>3.26</td>
<td>0.1</td>
</tr>
<tr>
<td>Del13q14 type II</td>
<td>6.93</td>
<td>&lt;0.01</td>
<td>Del13q14 type II</td>
<td>7.76</td>
<td>&lt;0.01</td>
<td>Del13q14 type II</td>
<td>4.16</td>
<td>0.04</td>
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<tr>
<td>CD38 positive</td>
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<td>0.03</td>
<td>CD38 positive</td>
<td>3.55</td>
<td>0.04</td>
<td>CD38 positive</td>
<td>3.02</td>
<td>0.09</td>
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<tr>
<td>IgV_i unmutated</td>
<td>1.46</td>
<td>0.6</td>
<td>IgV_i unmutated</td>
<td>1.67</td>
<td>0.58</td>
<td>IgV_i unmutated</td>
<td>1.79</td>
<td>0.45</td>
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<tr>
<td>ZAP-70 positive</td>
<td>1.83</td>
<td>0.39</td>
<td>ZAP-70 positive</td>
<td>1.25</td>
<td>0.74</td>
<td>ZAP-70 positive</td>
<td>1.05</td>
<td>0.95</td>
</tr>
<tr>
<td>B. Total complexity (three or more lesions)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>P53 status</td>
<td>19.55</td>
<td>&lt;0.01</td>
<td>Del17p</td>
<td>20.98</td>
<td>&lt;0.01</td>
<td>Cells % alive postirradiation</td>
<td>5.69</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Del11q</td>
<td>6.3</td>
<td>&lt;0.01</td>
<td>Del11q</td>
<td>5.02</td>
<td>0.01</td>
<td>Del11q</td>
<td>3.38</td>
<td>0.07</td>
</tr>
<tr>
<td>Del13q14 type II</td>
<td>3.06</td>
<td>0.05</td>
<td>Del13q14 type II</td>
<td>3.36</td>
<td>0.03</td>
<td>Del13q14 type II</td>
<td>2.39</td>
<td>0.17</td>
</tr>
<tr>
<td>CD38 positive</td>
<td>2.41</td>
<td>0.1</td>
<td>CD38 positive</td>
<td>2.27</td>
<td>0.12</td>
<td>CD38 positive</td>
<td>2.23</td>
<td>0.16</td>
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<tr>
<td>IgV_i unmutated</td>
<td>1.45</td>
<td>0.54</td>
<td>IgV_i unmutated</td>
<td>1.58</td>
<td>0.43</td>
<td>IgV_i unmutated</td>
<td>1.93</td>
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<td>ZAP-70 positive</td>
<td>1.62</td>
<td>0.42</td>
<td>ZAP-70 positive</td>
<td>1.2</td>
<td>0.75</td>
<td>ZAP-70 positive</td>
<td>1.28</td>
<td>0.72</td>
</tr>
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</table>

NOTE: Values for % cells alive postirradiation are for samples with any 50% absolute change in normalized viability. None of the variables included in the models were subjected to a hierarchical classification.

Table 3. Gene copy number status for Mre11, ATM, and H2AFX and genomic complexity scores as measured through 50K SNP arrays, normalized p-ATM/ATM and p-ATM/actin ratios, and p53 aberrancy status

<table>
<thead>
<tr>
<th>CLL #</th>
<th>Gene copy status</th>
<th>Genomic complexity</th>
<th>WB ratio of pATM to:</th>
<th>p53 Status</th>
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<tr>
<td></td>
<td>Mre11</td>
<td>ATM</td>
<td>H2AFX</td>
<td>Losses</td>
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<td>18</td>
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<td>1n</td>
<td>2n</td>
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<td>182</td>
<td>2n</td>
<td>1n</td>
<td>2n</td>
<td>3</td>
</tr>
</tbody>
</table>

Abbreviation: Wt, wild-type.
nonsynonymous SNPs that predicted for amino acid changes in Mre11a (Supplementary Fig. S3B), although effects of these changes on Mre11a remain undefined. Sequencing of the H2AFX coding exon did not disclose mutations and too few cases had H2AFX +/- status to reliably detect mRNA expression differences. No case was found not to express H2AFX.

Discussion

Genomic instability and elevated genomic complexity have been linked to poor outcome in multiple types of cancer (34, 35). In CLL, elevated genomic complexity exists in 15% to 30% of previously nontreated patients and has identified a substantial disease subset with an aggressive disease course (6). It therefore seemed important to establish a link between the identification of aggressive CLL, molecular defects in the DNA double-strand break response, and unbiased assessments of genomic complexity in CLL, all in the setting of prevailing therapies with genotoxic drugs as reported here for a large CLL cohort.

Using radiation as a relatively selective modality to introduce DNA double-strand breaks into CLL cells, we confirmed that a substantial subset of CLL displays relative or absolute radiation resistance; this is in the setting of p53 aberrations, as expected, as well as in CLL cases with wild-type p53 (36–38). Given our prior findings of general sensitivity of CLL with wild-type p53 to p53-induced apoptosis following treatment with Nutlin3-type MDM2 inhibitors, together, these data provide evidence for proximal defects in CLL in the signaling cascade that originates with DNA breaks and culminates in p53 activation. Additionally, and not further investigated in this study, CLL with wild-type p53 and radiation resistance may have elevated activity of NHEJ pathways that repair DNA damage before the execution of p53-dependent apoptosis, thus potentially contributing to the observed radiation resistance (39).

Using multivariate analysis, we found that resistance to radiation-induced apoptosis was a very strong independent predictor of genomic complexity in CLL, thus directly linking defective apoptotic DNA double-strand break
response with genomic complexity and aggressive CLL. Furthermore, we report for the first time a strong negative effect of impaired radiation-induced apoptosis on survival in CLL even in a subset of cases with wild-type p53.

Data presented here provide evidence for a strong effect of p53 aberrations on genomic complexity in CLL. This therefore corroborates findings of the negative prognostic effect of del17p (always associated with p53 aberrations) or rare isolated p53 mutations on CLL and explains why unbiased genomic complexity assessments generally capture the risk imparted on CLL through del17p (1, 6, 40). Our data furthermore identify del11q and the recently identified del13q14 type II deletions (Rb loss) as independent predictors of genomic complexity in CLL, providing independent evidence for the existence of additional defects in genes with effects on genomic stability in CLL.

Attempting to deconvolute the effect of del11q on genomic complexity, we measured ATM activity through ATM autophosphorylation postirradiation in ~140 CLL cases (41). To our knowledge, such an unbiased, comprehensive assessment of ATM activity in CLL has not been previously done. Measuring ATM activity as a variable in our genomic complexity analysis, rather than ATM mutations, seemed to be a more direct and specific way of interrogating ATM dysfunction as (a) the effect of individual mutations in large proteins are not always readily predictable, and (b) mutations and hypofunction in ATM can be dissociate. Multiple interesting findings emerged: (a) true functional ATM null states are rare in CLL, as suggested in prior studies (11); (b) ATM kinase displayed a continuum of activities across this large CLL cohort rather than discrete activity clusters, as exists for p53 (wild-type versus aberrant); (c) importantly, reduced ATM activity alone did not account for the effects of del11q on genomic complexity in CLL; (d) ATM activity, across the entire CLL cohort, was a modest predictor of genomic complexity and not independent of other dominant predictive factors as outlined above; and (e) ATM activity was not prognostic for CLL outcome. Together, these data based on a large CLL cohort are consistent with prior observations that monoallelic ATM states are sufficient to support a functional response to DNA double-strand breaks (11, 42). What remains unclear from this analysis is a mechanism for the reported moderately negative prognostic effect of ATM mutations in CLL, given our observation reported here that ATM hypoactivity seems to be only a minor contributor to genomic complexity in CLL and not predictive of outcome in CLL. One hypothesis is that a negative prognostic ATM effect is mostly driven by rare CLL cases with complete loss of ATM activity as suggested (which were rare in this cohort; ref. 10), or by compound defects in multiple closely linked pathogenetic genes on 11q as analyzed in this report (in the setting of low ATR expression; ref. 43). Finally, it remains formally possible that ATM autophosphorylation does not measure all critical ATM functions that are important to CLL biology.

Through analysis of precise boundaries of the deleted genomic regions as part of various interstitial 11q deletions, we quantify for the first time the frequent loss of either Mre11 or H2AFX in addition to invariant loss of ATM through del11q in CLL. Given the data from mouse models indicating the effects on genomic instability of each of these genes (although not yet fully explored in the case of Mre11a single copy loss), and data for strong synergistic effects on genomic instability of combined ATM and H2AFX defects, we surmise that del11q affects genomic stability in CLL through compound defects in genes important to genomic stability (44–47). These data therefore offer a possible explanation for the observed imperfect correlation between p53 dysfunction and ATM mutation rates in patients with del11q (48).

In this study, we have identified for the first time an independent contribution of del13q14 type II lesions on the genomic complexity in CLL. Recent data have suggested the existence of del13q14 subtypes, initially categorized as del13q14 type I (exclusive of Rb) and del13q14 type II (inclusive of Rb; ref. 32). Given the known effect of Rb null states on genomic instability in mouse models (49), Rb emerges as a candidate gene for genomic complexity in CLL. In addition, we note that (a) lower Rb mRNA levels were predictive of genomic complexity in CLL in univariate analysis (49), (b) that del13q14 type II lesions are associated with ~2.5-fold lower Rb mRNA levels than all other CLL cases; and (c) that lower Rb mRNA levels were weak (OR, ~1.5) but independent predictors of genomic complexity in models that did not include del13q14 type II lesions.

Although multiple other markers were predictors of genomic complexity in univariate analysis, only CD38 expression retained an independent value in multivariate models. Given that a CD38 polymorphism and, presumably, increased CD38 expression have recently been linked to CLL transformation to large cell lymphoma (Richter’s transformation), it will be interesting to determine whether elevated genomic complexity constitutes a risk factor for transformation as well (50).

In summary, our data provide solid support to the hypothesis that an impaired DNA double-strand break response due to multiple gene defects on multiple chromosomes is a major contributor to elevated genomic complexity in CLL, thus adding additional justification for the introduction of this novel biomarker into clinical practice and for the development of therapies not reliant on genotoxicity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Clinical Cancer Research

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