Inactivation of the CDKN2A Tumor-Suppressor Gene by Deletion or Methylation Is Common at Diagnosis in Follicular Lymphoma and Associated with Poor Clinical Outcome

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Abstract

Purpose: Follicular lymphoma, the most common indolent lymphoma, is clinically heterogeneous. CDKN2A encodes the tumor suppressors p16INK4a and p14ARF and frequently suffers deleterious alterations in cancer. We investigated the hypothesis that deletion or hypermethylation of CDKN2A might identify follicular lymphoma cases with distinct clinical or pathologic features potentially amenable to tailored clinical management.

Experimental Design: Deletion of CDKN2A was detected in pretreatment biopsy specimens using a single nucleotide polymorphism–based approach or endpoint PCR, and methylation of CpG elements in CDKN2A was quantified by methylation-specific PCR. Correlations between CDKN2A status and pathologic or clinical characteristics, including overall survival (OS), were investigated in 106 cases using standard statistical methods.

Results: Deletion of CDKN2A was detected in 9 of 111 samples (8%) and methylation was detectable in 22 of 113 (19%). CDKN2A was either deleted or methylated in 29 of 106 cases (27%) and this status was associated with inferior OS especially among patients treated with rituximab (P = 0.004). CDKN2A deletion or methylation was associated with more advanced age (P = 0.012) and normal hemoglobin (P = 0.05) but not with sex, FLIPI score, ECOG stage, LDH, performance status, number of involved nodal sites, B symptoms, histologic grade, the presence of a component of diffuse large B-cell lymphoma, proliferation index, or other pathologic factors.

Conclusions: Our results show that deletion or methylation of CDKN2A is relatively common in pretreatment follicular lymphoma biopsy specimens and defines a group of cases associated with reduced survival in the rituximab era presumably on the basis of more aggressive disease biology.

Introduction

Follicular lymphoma is common, accounting for approximately 20% to 30% of non-Hodgkin lymphoma (NHL), and its clinical course is typically indolent (1). Nonetheless, patients with follicular lymphoma experience reduced life expectancy with a median overall survival (OS) of approximately 10 years (2). Furthermore, some cases have a poor prognosis because of clinically progressive disease or histologic transformation to aggressive lymphoma, mostly diffuse large B-cell lymphoma (DLBCL; refs. 3 and 4). The relatively recent addition of rituximab, a chimeric monoclonal antibody directed at CD20, to therapeutic regimens used to treat follicular lymphoma has improved survival (2). The primary genetic event in most cases of follicular lymphoma is a somatic chromosomal translocation, t(14;18)(q32;q21), which results in the upregulation of the proto-oncogene BCL2 (5). However, t(14;18) is not sufficient to induce follicular lymphoma; secondary genetic alterations are required for full manifestation of the disease.

The CDKN2A genetic locus at human chromosome band 9p21 encodes the proteins p14ARF and p16INK4a, which are generated through alternative exon usage (Fig. 1; ref. 6). p14ARF activates p53 by rescuing it from proteosome-mediated proteolysis whereas p16INK4a antagonizes cyclin-dependent kinases 4 and 6 (CDK4/6), thereby blocking phosphorylation of the retinoblastoma protein pRb and, consequently, cell-cycle progression. The p53 and pRb signaling pathways are required for the induction of apoptosis or cellular senescence in response to a number of cellular stressors, including DNA damage and oncogene activation. Consistent with its role in the regulation of both
Inactivation of CDKN2A in Follicular Lymphoma

Translational Relevance

Follicular lymphoma, although generally indolent, is clinically heterogeneous in that some cases behave aggressively or are refractory to standard therapy. We find that deletion or CpG island methylation affecting the tumor-suppressor gene CDKN2A, ascertained in pretreatment follicular lymphoma biopsy specimens, is associated with reduced overall survival (P = 0.004) among patients who received rituximab as part of their therapeutic regimen, and that this association persists when FLIPI score and other conventional clinical or pathologic factors are added to a Cox proportional hazards model. In our dataset, CDKN2A deletion and methylation are complementary in predicting adverse outcome, underscoring the importance of ascertaining both genetic and epigenetic mechanisms when investigating tumor-suppressor genes as potential biomarkers. CDKN2A is inactivated more often by methylation than deletion in follicular lymphoma, suggesting that detecting CDKN2A methylation might identify candidates for therapy with methyltransferase inhibitors.

of these pathways, deleterious alterations of CDKN2A are prevalent in human cancers and play an important role in oncogenesis and tumor progression. Inactivation of CDKN2A in lymphoma can occur by gene deletion or, more frequently, methylation of CpG islands, whereas small deleterious mutations are relatively rare (7–10). Although deletions commonly eliminate all of the exons that encode p14ARF and p16INK4a, loss of p16INK4a may be the more important event in human cancers (11).

Deletion of CDKN2A occurs relatively commonly upon histologic transformation of follicular lymphoma to DBLCL, but has been considered rare in follicular lymphoma samples obtained before transformation (7, 9, 12–14). However, the recent application of technology capable of detecting homozygous deletions or copy-neutral LOH, also called acquired uniparental disomy (aUPD), has uncovered such alterations affecting CDKN2A in a substantial 12% to 20% of nontransformed follicular lymphoma samples (9, 15–17).

Methylation of cytosine residues in CpG islands in CDKN2A is associated with transcriptional silencing and therefore represents the functional equivalent of gene deletion (10, 18, 19). CDKN2A methylation is more prevalent in aggressive- than indolent-histology lymphomas and has been detected in up to 52% of follicular lymphoma samples (8, 10, 20, 21). Therefore, CpG island methylation is more common than gene deletion as a mechanism for inactivating CDKN2A in follicular lymphoma and seems to be an important step in tumor progression and transformation.

These considerations suggest the possibility that detecting deleterious alterations affecting the CDKN2A locus in pretreatment follicular lymphoma biopsy specimens might define a substantial subset of cases with aggressive underlying biology and clinical behavior, including a differential response to therapy. Indeed, a recent study reported an association between the presence of CDKN2A deletion or aUPD in diagnostic follicular lymphoma specimens and reduced OS (15). However, no study has yet evaluated the prognostic impact of gene methylation, the most prevalent mechanism of CDKN2A silencing in follicular lymphoma, in a large set of follicular lymphoma cases nor has the clinicopathological impact of CDKN2A silencing been evaluated in the rituximab era.

In this study, we have evaluated the clinicopathological associations of CDKN2A silencing ascertained at diagnosis in 106 cases of follicular lymphoma. We find that deletion or methylation of CDKN2A is associated with reduced OS and that this association is most striking when the analysis is restricted to the 62 patients who had been treated with rituximab.

Materials and Methods

Clinical samples

Cases were identified by searching the pathology and clinical records of Kingston General Hospital, Ontario,

Figure 1. Genomic configuration of the CDKN2A locus. Exons 1α, 2, and 3 of CDKN2A encode p16INK4a whereas exons 1β and 2 encode p14ARF; these different gene products are generated through differential pre-mRNA splicing and translation of exon 2 in alternative reading frames. CDKN2B encodes p15INK4b. The relative locations of 15 highly heterozygous SNPs used in this study are shown to scale, as are those of the CpG island used to ascertain methylation status and the sequences lying adjacent to exons 2 and 3 of CDKN2A that are the targets of the PCR primer sets used to ascertain homozygous deletions.

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Clin Cancer Res; 20(6) March 15, 2014 1677

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Canada. The following inclusion criteria were used: (i) availability of formalin-fixed, paraffin-embedded tissue samples (FFPE) from the original, diagnostic, pretreatment biopsy; and (ii) availability of baseline clinical and outcome data from clinical charts. Written approval for access to these clinical materials was obtained from the Health Sciences Research Ethics Board of Queen’s University.

Laser-capture microdissection and DNA extraction
Eight-millimeter sections of FFPE biopsy samples were mounted on uncharged glass slides and immunostained with an anti-CD20 antibody (Epitomics) using an automated immunostaining device as previously described to identify lymphoma cells (22). In cases where lymphoma cells congregated within lymphoid follicles, as usually occurred, entire follicles were collected by laser-capture microdissection (LCM); tissue from 3 to 10 lymphoid follicles, or an equivalent quantity of neoplastic tissue, was collected per case (Supplementary Fig. S1). DNA was purified from microdissected tissue using a commercial kit (QIAamp DNA Micro Kit; Qiagen Inc.) according to the manufacturer’s instructions and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.).

Single nucleotide polymorphism analysis for gene deletions affecting CDKN2A
Single nucleotide polymorphism (SNP) analysis was performed at the Analytical Genetics Technology Center at the University Health Network, Toronto, Canada, using the iPLEX Gold assay on the MassARRAY platform (Sequenom), as described (23). Fifteen highly polymorphic SNPs spanning a region of approximately 65 kb encompassing the CDKN2A locus were selected based on their heterozygosity rates in Utah residents with ancestry from northern and western Europe (CEU), as per the current release of the Human HapMap Project (Fig. 1; ref. 24). Primers for the multiplex PCR used in these assays were designed using the Sequenom MassARRAY designer software SpectroDesigner (Sequenom); primer sequences are shown in Supplementary Table S1. At least 10 ng/μL of DNA was used for each assay. For cases where all analyzed SNPs were homozygous, matching germline DNA samples from the same subject were purified from nonneoplastic tissue samples identified by searching the pathology archive. LOH was considered confirmed if the matching, nonneoplastic sample contained at least one heterozygous SNP. “No calls” for individual SNPs resulted from PCR failure due either to homozygous deletion involving CDKN2A or excessive DNA degradation. These possibilities were distinguished using endpoint PCR with primer sets that amplify regions adjacent to exons 2 and 3 of CDKN2A or control loci (Fig. 1 and Supplementary Table S2).

Methylation of a CpG island within CDKN2A
Genomic DNA was modified by the sodium bisulfite method using the EpiTect Bisulfite Kit (Qiagen) and then analyzed by quantitative PCR (qPCR) using the EpiTect MethyLight PCR Kit and the Hs_CDKN2A EpiTect MethyLight Assay (Qiagen) according to the manufacturer’s instructions (25, 26). This assay detects methylation at 2 CpG elements (Fig. 1; chr9:21974737-21974742 according to genome assembly GRCh37/hg19) that fall within a CpG island that begins 5′ of exon 10 of CDKN2A and extends into the coding region (chr9:21,974,579-21,975,306). The proportion of methylated DNA (\(C_{\text{meth}}\)) was calculated as a percentage using the formula 

\[ C_{\text{meth}} = \frac{C_{\text{TG}} - C_{\text{CTG}}}{100} \times \% \]

where \(C_{\text{TG}}\) represents the threshold cycle of the CG reporter (FAM channel) and \(C_{\text{TG}}\) represents the threshold cycle of the TG reporter (VIC channel). The assays were run on a Mastercycler ep realplex real-time PCR instrument (Eppendorf North America). Commercially obtained methylated versus unmethylated human DNA (Qiagen), and DNA purified from the Raji and HL60 human cell lines known to be completely methylated versus unmethylated, respectively, at the index CpG island were used to validate the assay (27).

Statistical analysis
The Fisher exact test was used to test the association between CDKN2A deletion or promoter methylation and clinicopathological variables. A Cox proportional hazards model was used to adjust for potential effects on the HR of the following variables: age, FLIPI score, histologic grade, and the presence of DLBCL. In addition to these prespecified variables, variables that were found to be asymmetrically distributed between the CDKN2A-deleted or CDKN2A-methylated cases (\(P < 0.15\)) were also added to the Cox model. Overall survival was determined from the time of diagnosis to the time of last follow-up or death and described by Kaplan–Meier with groups compared by the log-rank test. The Cox proportional hazards model was used to estimate adjusted and unadjusted HRs comparing mortality rates in Utah residents with ancestry from northern and western Europe (CEU) with an anti-CD20 antibody (Epitomics) using an automated immunostaining device as previously described to identify lymphoma cells (22).

Results
CDKN2A gene deletion
Genetic deletions affecting the CDKN2A locus were detected using an SNP-based approach with the Sequenom MassARRAY platform (23). Samples were considered to have retained heterozygosity if at least one of 15 highly heterozygous SNPs that span 65.4 kb of DNA encompassing the CDKN2A locus on chromosome 9 were found to be heterozygous in DNA from microdissected lymphoma samples (Fig. 1). Applying this assay to DNA purified from a group of established human cell lines with known CDKN2A status produced the expected results: Raji cells in which both alleles of CDKN2A are known to be intact were heterozygous at 3 of the 15 SNPs. HL60 cells that carry a hemizygous deletion involving CDKN2A were homozygous at all SNPs.
and K562 cells associated with homozygous CDKN2A deletion gave rise to no PCR products for SNP analysis (Fig. 2A; refs. 28 and 29). Also as expected, various SNPs were found to be homozygous in each of 5 samples of primary, non-neoplastic tonsillar lymphoid tissue.

This assay was applied to DNA purified from 118 pre-treatment follicular lymphoma biopsy samples in which lymphoma tissue had been enriched using LCM. The results demonstrated heterozygosity for at least one of the SNPs in 102 cases; these were therefore considered to be free of CDKN2A deletions (Fig. 2B). Of the remaining 16 cases, all SNPs were found to be homozygous in 12, whereas failed PCR reactions prevented SNP determinations in 4 (Fig. 2C). Of the 12 cases in which all of the SNPs were found to be homozygous, matching samples of germline DNA were retrievable from the hospital pathology archive in 9 cases and LOH was found to have occurred in 6 of these (Fig. 2C). The results were uninformative in the other 6 homozygous cases either because all SNPs were homozygous in the germline samples (3 cases) or because no matching germline sample was available (3 cases).

For the cases in which no results were obtained for any of the SNPs because of failed PCR reactions, the possibility of homozygous deletion of CDKN2A was investigated using endpoint PCR. Primers for intronic sequences adjacent to exons 2 and 3 of CDKN2A (denoted “Exon 2 PCR” or “Exon 3 PCR” in Fig. 1) were evaluated for their ability to amplify products of the expected size; homozygous CDKN2A deletion was considered to have occurred if no PCR product was obtained with either of the CDKN2A primer sets. PCR for genomic sequences associated with genes on different chromosomes (ACTB on chromosome 7 and GAPDH on chromosome 12) was used to control for DNA integrity. Applying this assay to DNA from cell lines produced the expected results: homozygous deletion of CDKN2A was detected in K562 cells but not HL60 or Raji cells (Fig. 2D). Applying the assay to the follicular lymphoma samples demonstrated homozygous CDKN2A deletions in 3 cases (case nos. 33, 72, and 197), all of which were among the 4 samples from which no SNP results had been obtainable (compare Fig. 2C and D). CDKN2A PCR products were obtained from the fourth case from which no SNP results were obtainable (case no. 12). This case was therefore considered uninformative with respect to CDKN2A deletion status.

In summary, of the 111 follicular lymphoma cases from which informative results were obtained, deletion of CDKN2A was demonstrable in 9 (8%); LOH occurred in 6 of these cases, consistent with either hemizygous deletion or aLOPD, and homozygous deletion occurred in 3.

**CDKN2A methylation analysis**

We determined the methylation status at 2 CpG elements within exon 1 of CDKN2A (Fig. 1) using quantitative methylation-specific PCR (MSP; refs. 25 and 26). The ability of this assay to detect methylation in a quantitative manner was verified using serial dilution of chemically methylated DNA into unmethylated DNA, DNA from cell lines of known methylation status at this locus, and DNA purified from 10 samples of hyperplastic tonsil tissue (Supplementary Table S3). The ages of the patients from whom the tonsil samples were obtained ranged from 17 to 66 (mean 27) years, or 17 to 35 years after excluding a 66-year-old outlier. The methylation status was evaluable in LCM-enriched follicular lymphoma samples from 113 cases. Methylation was detectable in 22 (19%) cases: methylation of from 1% to 80% of DNA was detected in 16 (14%) cases and methylation of greater than 80% was detectable in 6 (5%) cases (Fig. 3).

Examining the relative distribution of CDKN2A gene deletion, methylation, and high-level p16INK4a expression, as determined by immunohistochemistry (IHC) in a previous study, across our cases uncovered no cases with both CDKN2A gene deletion and abundant p16INK4a expression (Fig. 4; ref. 22). Two cases with abundant p16INK4a expression (nos. 135 and 188) showed low-level methylation of from 6% to 10% of DNA.

**Clinical data**

Baseline clinical and outcome data were available from 106 subjects from whom CDKN2A deletion and methylation status were ascertainable. Fifty-two subjects were female and 54 were male. The median age at diagnosis was 58 (range 34–89) years. Forty-two cases were histologic grade 1, 31 cases were grade 2, 32 cases were grade 3A, and 1 case was grade 3B. A tumor component that met the histologic criteria for DLBCL was appreciable in the biopsy sample in 25 cases; the associated follicular lymphoma component was grade 3A in 17 of these cases and grade 3B in 1 (30). Median follow-up time was 6.3 (range 0.15–16) years.

The subjects were diagnosed between 1996 and 2008. During this period rituximab alone followed by rituximab in combination with chemotherapy became standard treatment for follicular lymphoma, contributing to treatment heterogeneity among our subjects (Table 1; ref. 2). Nineteen of the patients received no systemic treatment and either remained on watchful waiting (15 patients) or received radiation treatment alone (4 patients). Of the 87 patients that received systemic treatment, 37 (43%) received rituximab alone followed by rituximab in combination with intravenous chemotherapy as first-line treatment, 26 (30%) received oral chemotherapy, 23 (26%) received cytotoxic chemotherapy without rituximab, and 1 received rituximab as a single agent. Forty-five subjects (52% of those who received chemotherapy) received a single treatment regimen, 16 (18%) received 2 regimens, and 26 (30%) received from 3 to 6 regimens. Six patients underwent autologous stem cell transplantation. Sixty-two patients (71% of those who received chemotherapy) received rituximab in combination with intravenous chemotherapy as first-line treatment, 26 (30%) received oral chemotherapy, 23 (26%) received cytotoxic chemotherapy without rituximab, and 1 received rituximab as a single agent. Forty-five subjects (52% of those who received chemotherapy) received a single treatment regimen, 16 (18%) received 2 regimens, and 26 (30%) received from 3 to 6 regimens. Six patients underwent autologous stem cell transplantation. Sixty-two patients (71% of those who received chemotherapy) received rituximab at some point in their treatment and 27 patients (31%) received rituximab maintenance therapy following rituximab-containing chemotherapy.

**CDKN2A silencing is associated with reduced OS**

No statistically significant effect on OS was evident for either CDKN2A gene deletion or methylation when these
Loss of heterozygosity

SNP results uninformative
No SNP data

Sample
RS2069418
RS1063192
RS3218020
RS78545330
RS7036656
RS3731211
RS3731239
RS2518719
RS3731257
RS67226702
RS7041637
RS10757261
RS10757260
RS10965197
RS4977750

CDKN2A exon 2
CDKN2A exon 3
ACTB
GAPDH

Sample
RS2069418
RS1063192
RS3218020
RS78545330
RS7036656
RS3731211
RS3731239
RS2518719
RS3731257
RS67226702
RS7041637
RS10757261
RS10757260
RS10965197
RS4977750

CDKN2A exon 2
CDKN2A exon 3
ACTB
GAPDH
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variables were examined individually, although trends toward reduced survival were apparent with a more pronounced effect for methylation (deletion, $P = 0.46$; methylation, $P = 0.19$; Supplementary Figs. S1B and S2A). However, combining cases with either of these alterations into a single "CDKN2A deleted or methylated" group showed that CDKN2A was affected by either deletion or methylation in 29 of 106 cases (27%) and uncovered a trend toward reduced OS that approached statistical significance ($P = 0.052$; Fig. 5A). We considered cases with at least 1% methylation as transcriptionally silenced for the purpose of this analysis. Because we did not directly evaluate the effect of CDKN2A methylation on gene transcription, and considering that reduced CDKN2A expression was demonstrated recently in cases of DLBCL with at least 25% methylation of the gene, we repeated the Kaplan–Meier analysis using 25% methylation status (22). Finally, because CDKN2A silencing and reduced p16INK4a expression is taken into account ($P = 0.017$; Fig. 5B). The presence of a dominant component of DLBCL in primary biopsies showing follicular lymphoma is associated with more aggressive disease (32). Therefore, we repeated the survival analysis after excluding cases that contained a DLBCL component and noted persistence of the association between CDKN2A silencing and reduced OS ($P = 0.043$; Fig. 5C). Finally, the adverse prognostic association was most pronounced when the analysis was limited to patients treated with rituximab ($P < 0.004$; Fig. 5D), although the ability of treatment with rituximab to modify the association between CDKN2A silencing and OS was not statistically significant (Wald test for interaction by the Cox PH model, $P = 0.17$).

The data from the subjects who had been treated with rituximab were then evaluated for associations between CDKN2A silencing and other features. With respect to clinical variables (Table 2), a negative association was apparent between CDKN2A silencing and low hemoglobin ($P = 0.05$) as was a trend toward an association between CDKN2A silencing and age at diagnosis of greater than 60 years ($P = 0.06$). The average age at diagnosis was 9.5 (95% CI, 2.1–16.9; $P = 0.012$) years more advanced in patients with silenced CDKN2A, with mean ages of 65.7 years versus 56.2 years. The adjusted log-rank test showed that the association between CDKN2A status and reduced OS remained statistically significant after stratifying by age ($P = 0.005$). The unadjusted HR estimated by the Cox proportional hazards model was 3.2 (95% CI, 1.4–7.5; $P = 0.0064$ by the Wald test) for patients with versus without silenced CDKN2A. After adding age (years), FLIPI (high or medium vs. low), histologic grade (1 or 2 versus 3), DLBCL (present vs. absent), and hemoglobin (normal vs. low) to the model, the estimated adjusted HR for CDKN2A status was 3.0 (1.1–8.0; $P = 0.027$). No statistically significant associations were noted between CDKN2A status and sex, FLIPI score, ECOG stage, LDH, performance status, number of involved nodal sites, the presence of B symptoms, histologic grade, the presence of a component of DLBCL, or the proliferation index (Table 3). We previously determined the prevalence in these cases of cells expressing the cell-cycle regulatory proteins p53, pRb, or cyclin D3 by IHC; no statistically significant associations were evident between these markers and CDKN2A status (22). Finally, because differences in treatment can contribute to differences in survival, it is noteworthy that we observed no statistically significant associations between CDKN2A status and receipt of either first-line treatment with chemotherapy plus rituximab or maintenance rituximab (Table 4).

Figure 2. Detection of genomic deletions involving CDKN2A. A, validation of the SNP-based assay using DNA from cell lines of known CDKN2A deletion status and samples of hyperplastic tonsils. Results for individual SNPs are in columns, whereas results for individual samples are in rows. Black, heterozygous; gray, homozygous; and, white, no data. Raji cells carry 2 intact alleles of CDKN2A, whereas HL60 and K562 cells carry hemizygous and homozygous CDKN2A deletions, respectively. T1–T5, samples of hyperplastic tonsil tissue from different subjects. B, follicular lymphoma samples with retained heterozygosity at CDKN2A. C, follicular lymphoma samples with either homozygosity or no data for all SNPs tested across the CDKN2A locus. D, L, lymphoma and N, matched nonneoplastic tissue. Results of endpoint PCR assay for homozygous deletion of CDKN2A. Primers for genomic sequences associated with the ACTB and GAPDH loci were used to control for DNA integrity. The 4 cases for which no SNP data were obtainable, as shown in part (C), are indicated with asterisks.

Figure 3. Quantification of CDKN2A methylation. The bins in the histogram correspond to percent methylation of tandem CpG elements associated with CDKN2A. The number above each bar indicates the number of cases in the corresponding bin.
Discussion

Inactivation of the CDKN2A locus in cancer cells can occur by deletion, small mutations, or epigenetic changes including DNA methylation. In this study, we concentrated on the most prevalent alterations, namely gene deletion and DNA methylation, with a view to uncovering clinicopathological correlates of potential clinical utility (7, 33, 34). Previous work has indicated that CDKN2A methylation is more prevalent than gene deletion in NHLs, including follicular lymphoma (10, 13, 20, 35). However, ours is by far the largest study to evaluate CDKN2A methylation in follicular lymphoma and the first to investigate its clinical implications in the rituximab era (8, 10, 21, 34–36). Using a sensitive and quantitative assay, we demonstrate the presence of at least some methylation of this locus in 22 of 113 cases (19%). Our observation that neither CDKN2A deletion nor methylation is significantly associated with reduced OS when considered in isolation but, when considered together, define a group of subjects with a significant reduction in OS indicates that the 2 markers are prognostically complementary in our dataset and supports the idea that they are equivalent relative to CDKN2A function. These findings underscore the importance of ascertaining both genetic and epigenetic mechanisms for gene silencing when evaluating the functional status of tumor-suppressor genes in cancers.

Table 1. Clinical management

<table>
<thead>
<tr>
<th>No systemic treatment</th>
<th>No.</th>
<th>Watchful waiting</th>
<th>15</th>
<th>Radiotherapy alone</th>
<th>4</th>
<th>Total</th>
<th>19</th>
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<tr>
<td>First-line chemotherapy</td>
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<td>% chemo pts.</td>
<td></td>
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<tr>
<td>R-CHOP, R-CVP, or R-CEOP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37</td>
<td>43</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral chemotherapy agent&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHOP, CVP, or CEOP</td>
<td>23</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Rituximab alone</td>
<td>1</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Total</td>
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<tr>
<td>Number of chemo regimens</td>
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<td>% pts.</td>
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<td>1</td>
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<td>30</td>
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<sup>a</sup>R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; R-CVP, rituximab, cyclophosphamide, vincristine, and prednisone; R-CEOP, rituximab, cyclophosphamide, etoposide, vincristine, and prednisone.

<sup>b</sup>Chlorambucil, 23 patients; cyclophosphamide, 2 patients; or, fludarabine, 1 patient.

Previous work has indicated that CDKN2A methylation is more prevalent than gene deletion in NHLs, including follicular lymphoma (10, 13, 20, 35). However, ours is by far the largest study to evaluate CDKN2A methylation in follicular lymphoma and the first to investigate its clinical implications in the rituximab era (8, 10, 21, 34–36). Using a sensitive and quantitative assay, we demonstrate the presence of at least some methylation of this locus in 22 of 113 cases (19%). Our observation that neither CDKN2A deletion nor methylation is significantly associated with reduced OS when considered in isolation but, when considered together, define a group of subjects with a significant reduction in OS indicates that the 2 markers are prognostically complementary in our dataset and supports the idea that they are equivalent relative to CDKN2A function. These findings underscore the importance of ascertaining both genetic and epigenetic mechanisms for gene silencing when evaluating the functional status of tumor-suppressor genes in cancers.

Our results are consistent with those of others in showing that deletion of CDKN2A is detectable at diagnosis in a substantial minority of nontransformed follicular lymphoma cases, including those with grade 1 or grade 2 histology. We found deletions in 9 (6 LOH, 3 homozygous deletions) of 111 (8%) primary diagnostic follicular lymphoma samples. This is somewhat lower than the 12% to 20% detected in recent studies in which array-based comparative genomic hybridization (aCGH) was applied to nontransformed follicular lymphoma specimens (9, 15–17). Our approach using a SNP-based assay was capable of detecting heterozygous deletions as small as 65.4 kb, which is comparable to the resolution achievable using 500K SNP arrays and smaller than the 430 kb minimally affected region across the CDKN2A/B locus reported by Schwaenen and colleagues.

Figure 4. Relative distribution across samples of alterations affecting CDKN2A. Columns correspond to individual cases. For deletion status, black indicates loss of heterozygosity or homozygous deletion. For methylation status, the degree of methylation is indicated by shades of gray or black, which correspond to those used in Fig. 3. For p16INK4a high status, black indicates abundant expression as determined by IHC and as defined in reference (22). Only cases from which data were available for all 3 parameters are included in the plot.
The relatively low apparent prevalence of CDKN2A deletions in our dataset may reflect the necessity to exclude 3 cases that were associated with SNP homozygosity from our analysis because matched germline samples were not available, our stringent requirement for all of the SNPs in the region of interest to be homozygous in order for LOH to be considered to have occurred, or our exclusive use of specimens from initial diagnostic biopsies. Conversely, our use of LCM to enrich for lymphoma cells should have militated against false-negative results because of infiltration by non-neoplastic cells, an issue that may be especially problematic in follicular lymphoma because of its characteristic cellular heterogeneity.

We demonstrate an association between CDKN2A silencing and advanced age at diagnosis. Multiple tumor-suppressor genes, including CDKN2A, become methylated with age in nonneoplastic tissues, raising the possibility that the association between CDKN2A silencing and reduced OS was an indirect effect of direct associations of each of these variables with advanced age (37). However, the association between CDKN2A silencing and reduced OS remained strong after stratifying by age in an adjusted log-rank test or incorporating age in a Cox proportional hazards model, consistent with a direct causal relationship between CDKN2A silencing and clinical outcome. It is possible that cells in older patients have had more time to acquire alterations that silence CDKN2A and that these then contribute to relatively aggressive disease behavior.

We showed recently that abundant expression of p16INK4a, as determined by conventional IHC, was associated with clinical and pathologic evidence of more aggressive disease, including reduced OS, elevated serum LDH, grade 3 histology, and the presence of an associated component of DLBCL (22). The association between abundant p16INK4a expression and aggressive disease seemed discordant with the previously demonstrated associations between CDKN2A deletion and histologic transformation in follicular lymphoma or between CDKN2A methylation and aggressive-histology NHLs (7, 8, 12, 15). The current results provide an opportunity to consider the relationship that may exist between p16INK4a expression, CDKN2A silencing by deletion or methylation, and clinical and pathologic characteristics. In our dataset, abundant p16INK4a expression occurs in a set of 8 cases that are largely not deleted or methylated.
nonoverlapping with the 24 cases associated with CDKN2A deletion/LOH or methylation (Fig. 4). It is noteworthy that in the 2 cases that show both abundant staining for p16 INK4a and CDKN2A methylation, methylated DNA is of low relative abundance (6% and 11%) such that, had a higher cut-off value of 25% been used to designate cases as “methylated” at CDKN2A, as discussed above, there would have been no overlap between the p16 INK4a-high and CDKN2A-methylated groups. These considerations suggest that abundant p16 INK4a expression and CDKN2A silencing tend to be mutually exclusive in follicular lymphoma, as expected, and that the presence of either perturbation is associated with more aggressive disease.

The association between abundant p16 INK4a expression and more aggressive cancers has precedence in a variety of tumor types (reviewed in reference; ref. 38). B cells are exquisitely susceptible to oncogene-induced senescence (OIS) mediated by induction of CDKN2A (39). Expression of p16 INK4a upon oncogene activation is expected to subject cells to cytostatic effects, thereby making CDKN2A preferentially susceptible to subsequent inactivation during the Darwinian process of tumor progression. Thus, the presence of abundant p16 INK4a could conceivably reflect a particular stage of lymphomagenesis that exists after certain oncogenic events but before CDKN2A silencing. However, expression of p16 INK4a has been observed to increase progressively from normal tissue to fully malignant neoplasms, and associations between p16 INK4a expression and poor clinical outcomes have been reported in several cancer types (40–44). Upregulation of p16 INK4a in these scenarios could reflect deleterious alterations affecting pRb or perhaps other

### Table 2. Clinical factors and CDKN2A status

<table>
<thead>
<tr>
<th>CDKN2A not silenced</th>
<th>CDKN2A silenced</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>32 (52%)</td>
<td>5 (8.1%)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>16 (26%)</td>
<td>9 (15%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>25 (40%)</td>
<td>8 (13%)</td>
</tr>
<tr>
<td>Female</td>
<td>23 (37%)</td>
<td>6 (9.7%)</td>
</tr>
<tr>
<td>FLIPI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low/intermediate</td>
<td>30 (48%)</td>
<td>7 (11%)</td>
</tr>
<tr>
<td>High</td>
<td>18 (29%)</td>
<td>7 (11%)</td>
</tr>
<tr>
<td>LDH&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>31 (55%)</td>
<td>12 (21%)</td>
</tr>
<tr>
<td>High</td>
<td>11 (20%)</td>
<td>2 (3.6%)</td>
</tr>
<tr>
<td>ECOG stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I or II</td>
<td>14 (23%)</td>
<td>4 (6.5%)</td>
</tr>
<tr>
<td>III or IV</td>
<td>34 (55%)</td>
<td>10 (16%)</td>
</tr>
<tr>
<td>Hemoglobin&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>36 (59%)</td>
<td>14 (23%)</td>
</tr>
<tr>
<td>Low</td>
<td>11 (18%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Performance status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤1</td>
<td>43 (69%)</td>
<td>10 (16%)</td>
</tr>
<tr>
<td>&gt;1</td>
<td>5 (8.1%)</td>
<td>4 (6.5%)</td>
</tr>
<tr>
<td>No. of nodal sites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤4</td>
<td>30 (48%)</td>
<td>6 (9.7%)</td>
</tr>
<tr>
<td>&gt;4</td>
<td>18 (29%)</td>
<td>8 (13%)</td>
</tr>
<tr>
<td>B symptoms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>37 (60%)</td>
<td>10 (16%)</td>
</tr>
<tr>
<td>Yes</td>
<td>11 (18%)</td>
<td>4 (6.5%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>All P values are calculated using the Fisher exact test.

<sup>b</sup>Elevated LDH, more than 200 U/L.

<sup>c</sup>Low hemoglobin, less than 120 g/L.

### Table 3. Pathologic factors and CDKN2A status

<table>
<thead>
<tr>
<th>CDKN2A not silenced</th>
<th>CDKN2A silenced</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histologic grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 or 2</td>
<td>32 (52%)</td>
<td>9 (15%)</td>
</tr>
<tr>
<td>3</td>
<td>16 (26%)</td>
<td>5 (8%)</td>
</tr>
<tr>
<td>DLBCL component present in biopsy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>40 (65%)</td>
<td>10 (16%)</td>
</tr>
<tr>
<td>Yes</td>
<td>8 (13%)</td>
<td>4 (6.5%)</td>
</tr>
<tr>
<td>Proliferation index&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>32 (53%)</td>
<td>10 (17%)</td>
</tr>
<tr>
<td>High</td>
<td>15 (25%)</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>p53 high</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>45 (75%)</td>
<td>13 (22%)</td>
</tr>
<tr>
<td>Yes</td>
<td>2 (3.3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>pRb low</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>38 (73%)</td>
<td>9 (17%)</td>
</tr>
<tr>
<td>Yes</td>
<td>3 (5.8%)</td>
<td>2 (3.9%)</td>
</tr>
<tr>
<td>Cyclin D3 high</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>20 (35%)</td>
<td>5 (8.8%)</td>
</tr>
<tr>
<td>Yes</td>
<td>25 (44%)</td>
<td>7 (12%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>All P values are calculated using the Fisher exact test.

<sup>b</sup>High proliferation index, Ki-67 staining in more than 50% of cells.

### Table 4. Chemotherapy and CDKN2A status

<table>
<thead>
<tr>
<th>CDKN2A not silenced</th>
<th>CDKN2A silenced</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-line chemo + rituximab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>19 (31%)</td>
<td>6 (9.7%)</td>
</tr>
<tr>
<td>Yes</td>
<td>29 (47%)</td>
<td>8 (13%)</td>
</tr>
<tr>
<td>Maintenance rituximab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>25 (40%)</td>
<td>10 (16%)</td>
</tr>
<tr>
<td>Yes</td>
<td>23 (37%)</td>
<td>4 (6.5%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>All P values are calculated using the Fisher exact test.
mediators of p16\(^{INK4a}\) or pRb signaling. Indeed, a reciprocal relationship has been observed between p16\(^{INK4a}\) expression and pRb function in several cancers (38). The mechanism linking p16\(^{INK4a}\) induction with loss of pRb function has not been elucidated definitively but could involve escape of CDKN2A from pRb-mediated transcriptional silencing (45). Having recently quantified pRb by IHC in our follicular lymphoma cases, we were able to examine our data for potential associations between the abundance of p16\(^{INK4a}\) and pRb (22). Although the availability of longer clinical follow-up allowed us to demonstrate a statistically significant association between reduced pRb staining and OS (\(P = 0.039\); Supplementary Fig. S3), the hypothesized association between elevated p16\(^{INK4a}\) expression and relatively poor clinical outcome in follicular lymphoma remains undetermined.

We found that the association of CDKN2A silencing with reduced OS was strengthened when the analysis was limited to patients treated with rituximab. This suggests that loss of CDKN2A function contributes to more aggressive follicular lymphoma biology in the context of contemporary clinical management and raises the possibility that CDKN2A mediates some of the toxic effects of rituximab on follicular lymphoma B cells. Our findings are consistent with the recently described association between CDKN2A deletion in DLBCL, another follicle center cell-derived lymphoma, with resistance to R-CHOP therapy (46, 47). In most of our CDKN2A “silenced” cases, CDKN2A is methylated but not deleted (Fig. 4). DNA methyltransferase inhibitors such as azacitidine or decitabine are thought to block the conservation of methylation in newly replicated DNA and are effective in the treatment of myeloid neoplasms (48–50). Furthermore, silenced CDKN2A could identify instances of follicular lymphoma that are particularly dependent upon CDK4/6 function and therefore amenable to treatment with CDK inhibitors (51). Especially given these clinical implications, it will be important to validate our findings using an independent cohort, preferably one in which patients were managed according to a standard, contemporary treatment protocol.

In summary, we find that silencing of CDKN2A by gene deletion or methylation is relatively common in primary, diagnostic follicular lymphoma samples and portends inferior clinical outcome. Further study is warranted to elucidate the mechanisms that determine the clinical behavior of CDKN2A-silenced cases and the implications for clinical management.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A. Alhejaily, H.E. Feilotter, T. Baetz, D.P. LeBrun
Development of methodology: A. Alhejaily, H.E. Feilotter, T. Baetz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Alhejaily, T. Baetz, D.P. LeBrun
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Alhejaily, A.G. Day, H.E. Feilotter, T. Baetz
Writing, review, and/or revision of the manuscript: A. Alhejaily, A.G. Day, H.E. Feilotter, T. Baetz, D.P. LeBrun
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Alhejaily, D.P. LeBrun
Study supervision: H.E. Feilotter, D.P. LeBrun

Acknowledgments

The authors thank Dr. X. Liu and Ms. C. Crozier in the Analytical Genetics Technology Centre at University Health Network, Toronto, Canada, for technical assistance with SNP genotyping, and Dr. C. Tayade, Queen’s University, Kingston, Ontario, Canada, for giving us access to a laser capture microdissection instrument.

Grant Support

This work was supported by a grant to D.P. LeBrun from the Leukemia and Lymphoma Society of Canada. The contribution to this work by A. Alhejaily was supported by King Fahad Medical City (KFMC) through the Saudi Arabian Cultural Bureau, Ottawa, Canada.

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Received August 7, 2013; revised November 13, 2013; accepted January 4, 2014; published OnlineFirst January 21, 2014.

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www.aacrjournals.org Published OnlineFirst January 21, 2014; DOI: 10.1158/1078-0432.CCR-13-2175

Clin Cancer Res; 20(6) March 15, 2014 1685

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