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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Translational Relevance

Follicular lymphoma (FL), 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 pre-treatment FL 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 pathological 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.
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Abstract

Purpose: Follicular lymphoma (FL), 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 FL cases with distinct clinical or pathological features potentially amenable to tailored clinical management.

Experimental Design: Deletion of CDKN2A was detected in pre-treatment biopsy specimens using a SNP-based approach or endpoint PCR, and methylation of CpG elements in CDKN2A was quantified by methylation-specific PCR. Correlations between CDKN2A status and pathological or clinical characteristics, including overall survival, 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 out of 106 cases (27%) and this status was associated with inferior overall survival (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, histological grade, the presence of a component of diffuse large B-cell lymphoma, proliferation index or other pathological factors.

Conclusions: Our results show that deletion or methylation of CDKN2A is relatively common in pre-treatment FL 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.
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Introduction

Follicular lymphoma (FL) is common, accounting for approximately 20-30% of non-Hodgkin lymphoma (NHL), and its clinical course it typically indolent [1]. Nonetheless, patients with FL 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 histological transformation to aggressive lymphoma, mostly diffuse large B-cell lymphoma (DLBCL) [3, 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 FL is a somatic chromosomal translocation, t(14;18)(q32;q21), which results in the up-regulation of the proto-oncogene BCL2 [5]. However, t(14:18) is not sufficient to induce FL; 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) [6]. p14ARF activates p53 by rescuing it from proteosome-mediated proteolysis while 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 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...
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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 histological transformation of FL to DBLCL but has been considered rare in FL samples obtained prior to transformation [7, 9, 12-14]. However, the recent application of technology capable of detecting homozygous deletions or copy-neutral loss of heterozygosity (LOH), also called acquired uniparental disomy (aUPD), has uncovered such alterations affecting **CDKN2A** in a substantial 12 to 20% of non-transformed FL 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 FL samples [8, 10, 20, 21]. Therefore, CpG island methylation is more common than gene deletion as a mechanism for inactivating **CDKN2A** in FL and appears to be an important step in tumor progression and transformation.

These considerations suggest the possibility that detecting deleterious alterations affecting the **CDKN2A** locus in pre-treatment FL biopsy specimens might define a substantial subset of cases with aggressive underlying biology and clinical behaviour, including a differential response to therapy. Indeed, a recent study reported an association between the presence of **CDKN2A** deletion or aUPD in diagnostic FL 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 FL, in a large set of FL cases nor has the clinicopathological impact of CDKN2A silencing been evaluated in the rituximab era.

In the current study we have evaluated the clinicopathological associations of CDKN2A silencing ascertained at diagnosis in 106 cases of FL. 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.
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Materials and methods

Clinical samples

Cases were identified by searching the pathology and clinical records of Kingston General Hospital, Ontario, Canada. The following inclusion criteria were used: 1) availability of formalin-fixed, paraffin-embedded tissue samples (FFPE) from the original, diagnostic, pre-treatment biopsy; and, 2) 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, Burlingame, CA) 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 (Supplemental Fig. S1). DNA was purified from microdissected tissue using a commercial kit (QIAamp DNA Micro Kit, QIAGEN Inc, Toronto, Canada) according to the manufacturer's instructions and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc, Mississauga, Ontario, Canada).

Single nucleotide polymorphism analysis for gene deletions affecting CDKN2A

Single nucleotide polymorphism (SNP) analysis was performed at the Analytical Genetics...
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Technology Center at the University Heath Network, Toronto, Canada, using the iPLEX Gold assay on the MassARRAY platform (Sequenom, San Diego, CA), 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) [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 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 non-neoplastic tissue samples identified by searching the pathology archive. Loss of heterozygosity (LOH) was considered confirmed if the matching, non-neoplastic 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 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 1α of CDKN2A and extends into the coding region.
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(chr9:21,974,579-21,975,306). The proportion of methylated DNA (Cmeth) was calculated as a percentage using the formula Cmeth = 100/[1+2(CtCG - CtTG)]%, where CtCG represents the threshold cycle of the CG reporter (FAM channel) and CtTG 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, Toronto, Ontario, Canada). 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

Fisher’s 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 hazards ratio (HR) of the following variables: age, FLIPI score, histological grade and the presence of DLBCL. In additional to these pre-specified variables, variables that were found to be asymmetrically distributed between the CDKN2A-deleted or -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 hazard ratios comparing mortality between patients with and without CDKN2A deletion or methylation and to perform a test for interaction to assess if this hazard ratio differed significantly in patients treated by rituximab. Cases with missing SNP-LOH or methylation data were dropped from this analysis. A P-value less than 0.05 was considered statistically significant, and no adjustment was made for multiplicity of tests. Statistical analysis
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Results

CDKN2A gene deletion

Genetic deletions affecting the CDKN2A locus were detected using a 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) [28, 29]. Also as expected, various SNPs were found to be heterozygous in each of 5 samples of primary, non-neoplastic tonsillar lymphoid tissue.

This assay was applied to DNA purified from 118 pre-treatment FL 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 due to 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 *CKDN2A* 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 FL 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 Figs 2C and 2D). *CKDN2A* 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 FL 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 aUPD, and homozygous deletion occurred in 3.

**CDKN2A methylation analysis**

We determined the methylation status at 2 CpG elements within exon 1α of *CDKN2A* (Fig. 1)
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using quantitative methylation-specific PCR (MSP) [25, 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 (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 FL 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 p16\(^{\text{INK4a}}\) expression, as determined by immunohistochemistry (IHC) in a previous study, across our cases uncovered no cases with both *CDKN2A* gene deletion and abundant p16\(^{\text{INK4a}}\) expression (Fig. 4) [22]. Two cases with abundant p16\(^{\text{INK4a}}\) 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 histological grade 1, 31 cases were grade 2, 32 cases were grade 3A and 1 case was grade 3B. A tumor component that met the histological criteria for DLBCL was appreciable in the biopsy sample in 25 cases; the associated FL 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 FL, contributing to treatment heterogeneity among our subjects (Table 1) [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 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 (ASCT). 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 overall survival**

No statistically significant effect on OS was evident for either CDKN2A gene deletion or methylation when these variables were examined individually, although trends towards reduced survival were apparent with a more pronounced effect for methylation (deletion, \( P=0.46 \); methylation, \( P=0.19 \). Supplemental Figs S2A and S1B). 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 out of 106 cases (27%) and uncovered a
trend towards 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. Since 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 as a cutpoint and demonstrated a statistically significant reduction in OS among CDKN2A-"silenced" cases ($P=0.025$, data not shown) [31]. We showed previously that detection of high-level expression of p16$^{INK4a}$ by IHC is associated with inferior OS in FL [22]. We now observe that the prognostic impact of CDKN2A deletion/methylation status persists when p16$^{INK4a}$ expression is taken into account ($P=0.017$, Fig. 5B). The presence of a dominant component of DLBCL in primary biopsies showing FL 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 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 towards 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 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 hazard ratio estimated by the Cox proportional hazards model was 3.2 (95% CI, 1.4-7.5; $P=0.0064$ by Wald test) for patients with versus without silenced $CDKN2A$. After adding age (years), FLIPI (high or medium versus low), histological grade (1 or 2 versus 3), DLBCL (present versus absent) and hemoglobin (normal versus low) to the model, the estimated adjusted hazard ratio 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, histological grade, the presence of a component of DLBCL, or the proliferation index. We previously determined the prevalence in these cases of cells expressing the cell cycle regulatory proteins p53, pRb or cyclin D3 by immunohistochemistry; no statistically significant associations were evident between these markers and $CDKN2A$ status [22]. Finally, since 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).
Discussion

Inactivation of the \textit{CDKN2A} locus in cancer cells can occur by deletion, small mutations, or epigenetic changes including DNA methylation. In the current 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 \textit{CDKN2A} methylation is more prevalent than gene deletion in NHLs including FL [10, 13, 20, 35]. However, ours is by far the largest study to evaluate \textit{CDKN2A} methylation in FL 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 out of 113 cases (19%). Our observation that neither \textit{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 two markers are prognostically complementary in our dataset and supports the idea that they are equivalent relative to \textit{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 \textit{CDKN2A} is detectable at diagnosis in a substantial minority of non-transformed FL cases, including those with grade 1 or 2 histology. We found deletions in 9 (6 LOH, 3 homozygous deletions) out of 111 (8%) primary diagnostic FL 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 non-transformed FL 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 [15]. 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 due to infiltration by non-neoplastic cells, an issue that may be especially problematic in FL due to 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 non-neoplastic 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
We showed recently that abundant expression of p16\(^{\text{INK4a}}\), as determined by conventional IHC, was associated with clinical and pathological 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 p16\(^{\text{INK4a}}\) expression and aggressive disease seemed discordant with the previously demonstrated associations between CDKN2A deletion and histological transformation in FL 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 p16\(^{\text{INK4a}}\) expression, CDKN2A silencing by deletion or methylation, and clinical and pathological characteristics. In our dataset, abundant p16\(^{\text{INK4a}}\) expression occurs in a set of 8 cases that are largely non-overlapping 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\(^{\text{INK4a}}\) 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\(^{\text{INK4a}}\)-high and CDKN2A-methylated groups. These considerations suggest that abundant p16\(^{\text{INK4a}}\) expression and CDKN2A silencing tend to be mutually exclusive in FL, as expected, and that the presence of either perturbation is associated with more aggressive disease.

The association between abundant p16\(^{\text{INK4a}}\) expression and more aggressive cancers has precedence in a variety of tumor types (reviewed in reference [38]). B-cells are exquisitely susceptible to oncogene-induced senescence (OIS) mediated by induction of CDKN2A [39].
Expression of p16\textsuperscript{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\textsuperscript{INK4a} could conceivably reflect a particular stage of lymphomagenesis that exists after certain oncogenic events but prior to CDKN2A silencing. However, expression of p16\textsuperscript{INK4a} has been observed to increase progressively from normal tissue to fully malignant neoplasms, and associations between p16\textsuperscript{INK4a} expression and poor clinical outcomes have been reported in several cancer types [40-44]. Up-regulation of p16\textsuperscript{INK4a} in these scenarios could reflect deleterious alterations affecting pRb or perhaps other mediators of p16\textsuperscript{INK4a} or pRb signaling. Indeed, a reciprocal relationship has been observed between p16\textsuperscript{INK4a} expression and pRb function in several cancers [38]. The mechanism linking p16\textsuperscript{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 FL cases, we were able to examine our data for potential associations between the abundance of p16\textsuperscript{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 overall survival (P=0.039, Supplemental Fig. S3), the hypothetically predicted association between p16\textsuperscript{INK4a}-high status and reduced pRb staining was not observed (Supplemental Table S4). Therefore, the mechanism that underlies the association between elevated p16\textsuperscript{INK4a} expression and relatively poor clinical outcome in FL 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
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function contributes to more aggressive FL biology in the context of contemporary clinical management and raises the possibility that \textit{CDKN2A} mediates some of the toxic effects of rituximab on FL B-cells. Our findings are consistent with the recently-described association between \textit{CDKN2A} deletion in DLBCL, another follicle center cell-derived lymphoma, with resistance to R-CHOP therapy [46, 47]. In most of our \textit{CDKN2A-"silenced"} cases, \textit{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 \textit{CDKN2A} could identify instances of FL 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 \textit{CDKN2A} by gene deletion or methylation is relatively common in primary, diagnostic FL samples and portends inferior clinical outcome. Further study is warranted to elucidate the mechanisms that determine the clinical behaviour of \textit{CDKN2A}-silenced cases and the implications for clinical management.
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**Figure legends**

**Figure 1.** Genomic configuration of the $CDKN2A$ locus. Exons $\alpha$, 1, and 3 of $CDKN2A$ encode p16$^{INK4a}$ while exons $\beta$ and 2 encode p14$^{ARF}$; these different gene products are generated through differential pre-mRNA splicing and translation of exon 2 in alternative reading frames. $CDKN2B$ encodes p15$^{INK4b}$. 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.

**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; grey, 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. T1-5, 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; N, matched non-neoplastic 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.

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 grey or black, which correspond to those used in Figure 3. For p16INK4a high status, black indicates abundant expression as determined by immunohistochemistry and as defined in reference [22]. Only cases from which data were available for all 3 parameters are included in the plot.

Figure 5. Deletion or methylation of CDKN2A is associated with reduced overall survival. (A) Overall survival according to CDKN2A status in all cases. (B) Overall survival of all cases with p16INK4A expression status taken into account; two cases with both high p16INK4A expression and low-level CDKN2A gene methylation (6 and 10%) were included among the p16INK4A-high group for this analysis. (C) Overall survival in cases without a component of diffuse large B-cell lymphoma. (D) Overall survival in cases treated with rituximab.
Table 1. Clinical management

<table>
<thead>
<tr>
<th>No systemic treatment</th>
<th>No.</th>
<th></th>
</tr>
</thead>
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<tr>
<td>Watchful waiting</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Radiotherapy alone</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>First-line chemotherapy</th>
<th>No.</th>
<th>% chemo pts.</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-CHOP, R-CVP or R-CEOP</td>
<td>37</td>
<td>43</td>
</tr>
<tr>
<td>Oral chemotherapy agent</td>
<td>26</td>
<td>30</td>
</tr>
<tr>
<td>CHOP, CVP or CEOP</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td>Rituximab alone</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>Total</td>
<td>87</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of chemo regimens</th>
<th>No.</th>
<th>% pts.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>≥3</td>
<td>26</td>
<td>30</td>
</tr>
</tbody>
</table>

a R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone; R-CVP, rituximab, cyclophosphamide, vincristine and prednisone; R-CEOP, rituximab, cyclophosphamide, etoposide, vincristine and prednisone.

b Chlorambucil, 23 patients; cyclophosphamide, 2 patients; or, fludarabine, 1 patient.
Table 2. Clinical factors and CDKN2A status

<table>
<thead>
<tr>
<th></th>
<th>CDKN2A not silenced</th>
<th>CDKN2A silenced</th>
<th>P (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>32</td>
<td>5</td>
<td>0.06</td>
</tr>
<tr>
<td>&gt;60</td>
<td>16</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>25</td>
<td>8</td>
<td>0.77</td>
</tr>
<tr>
<td>Female</td>
<td>23</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td><strong>FLIPI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low/Intermediate</td>
<td>30</td>
<td>7</td>
<td>0.54</td>
</tr>
<tr>
<td>High</td>
<td>18</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td><strong>LDH (^a)</strong></td>
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<tr>
<td>Normal</td>
<td>31</td>
<td>12</td>
<td>0.48</td>
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<tr>
<td>High</td>
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<td>2</td>
<td></td>
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<tr>
<td><strong>ECOG stage</strong></td>
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<td></td>
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</tr>
<tr>
<td>I or II</td>
<td>14</td>
<td>4</td>
<td>1.0</td>
</tr>
<tr>
<td>III or IV</td>
<td>34</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Hemoglobin (^b)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>36</td>
<td>14</td>
<td>0.05</td>
</tr>
<tr>
<td>Low</td>
<td>11</td>
<td>0</td>
<td></td>
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<tr>
<td><strong>Performance status</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>≤1</td>
<td>43</td>
<td>10</td>
<td>0.19</td>
</tr>
<tr>
<td>&gt;1</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>No. of nodal sites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤4</td>
<td>30</td>
<td>6</td>
<td>0.23</td>
</tr>
<tr>
<td>&gt;4</td>
<td>18</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td><strong>B symptoms</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>37</td>
<td>10</td>
<td>0.73</td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Elevated LDH, more than 200 U/L.

\(^b\) Low hemoglobin, less than 120 g/L.

\(^c\) All P-values are calculated using Fisher's exact test.
Table 3. Pathological factors and CDKN2A status

<table>
<thead>
<tr>
<th></th>
<th>CDKN2A not silenced</th>
<th>CDKN2A silenced</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histologic grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 or 2</td>
<td>32 52%</td>
<td>9 15%</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>16 26%</td>
<td>5 8%</td>
<td></td>
</tr>
<tr>
<td><strong>DLBCL component present in biopsy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>40 65%</td>
<td>10 16%</td>
<td>0.44</td>
</tr>
<tr>
<td>Yes</td>
<td>8 13%</td>
<td>4 6.5%</td>
<td></td>
</tr>
<tr>
<td><strong>Proliferation index&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>32 53%</td>
<td>10 17%</td>
<td>0.74</td>
</tr>
<tr>
<td>High</td>
<td>15 25%</td>
<td>3 5%</td>
<td></td>
</tr>
<tr>
<td><strong>p53 high</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>45 75%</td>
<td>13 22%</td>
<td>1.0</td>
</tr>
<tr>
<td>Yes</td>
<td>2 3.3%</td>
<td>0 0%</td>
<td></td>
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<tr>
<td><strong>pRb low</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No</td>
<td>38 73%</td>
<td>9 17%</td>
<td>0.28</td>
</tr>
<tr>
<td>Yes</td>
<td>3 5.8%</td>
<td>2 3.9%</td>
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<tr>
<td><strong>Cyclin D3 high</strong></td>
<td></td>
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<tr>
<td>No</td>
<td>20 35%</td>
<td>5 8.8%</td>
<td>1.0</td>
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<tr>
<td>Yes</td>
<td>25 44%</td>
<td>7 12%</td>
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</tbody>
</table>

<sup>a</sup> High proliferation index, Ki-67 staining in more than 50% of cells.
<sup>b</sup> All P-values are calculated using Fisher's exact test.
<table>
<thead>
<tr>
<th></th>
<th>CDKN2A not silenced</th>
<th>CDKN2A silenced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td><strong>First-line chemo + rituximab</strong></td>
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<tr>
<td>No</td>
<td>19</td>
<td>31%</td>
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<tr>
<td>Yes</td>
<td>29</td>
<td>47%</td>
</tr>
<tr>
<td><strong>Maintenance rituximab</strong></td>
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<tr>
<td>No</td>
<td>25</td>
<td>40%</td>
</tr>
<tr>
<td>Yes</td>
<td>23</td>
<td>37%</td>
</tr>
</tbody>
</table>

*All P-values are calculated using Fisher's exact test.*
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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