Silencing of the p18\textsuperscript{INK4c} gene by promoter hypermethylation in the Reed-Sternberg cells in Hodgkin Lymphomas

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ABSTRACT

$p18^{INK4c}$ is a cyclin-dependent kinase inhibitor that interferes with the Rb-kinase activity of CDK6/CDK4. Disruption of $p18^{INK4c}$ in mice impairs B-cell terminal differentiation and confers increased susceptibility to tumor development; however, alterations of $p18^{INK4c}$ in human tumors have rarely been described. We used a tissue-microarray approach to analyze $p18^{INK4c}$ expression in 316 Hodgkin Lymphomas (HL). Nearly half of the HL cases showed absence of $p18^{INK4c}$ protein expression by RS cells, in contrast with the regular expression of $p18^{INK4c}$ in normal germinal center cells. To investigate the cause of $p18^{INK4c}$ repression in RS cells, the methylation status of the $p18^{INK4c}$ promoter was analyzed by methylation-specific PCR and bisulfite sequencing. Hypermethylation of the $p18^{INK4c}$ promoter was detected in 2/4 HL-derived cell lines, but in none of 7 NHL-derived cell lines. We also detected $p18^{INK4c}$ hypermethylation, associated with absence of protein expression, in 5/26 HL tumors. The correlation of $p18^{INK4c}$ immunostaining with the follow-up of the patients showed shorter overall survival in negative cases, independent of the International Prognostic Score. These findings suggest that $p18^{INK4c}$ may function as a tumor suppressor gene in HL, and its inactivation may contribute to the cell cycle deregulation and defective terminal differentiation characteristic of the RS cells.
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INTRODUCTION

Cyclin-dependent kinase inhibitors (CKIs) are a group of low molecular weight proteins that associate with cyclin-dependent kinases (CDKs), blocking their activity. The INK4 family of CKIs is comprised of $p16^{\text{INK4a}}$, $p15^{\text{INK4b}}$, $p18^{\text{INK4c}}$ and $p19^{\text{INK4d}}$, which specifically bind and inhibit CDK4 and CDK6, thereby preventing cyclin D-dependent phosphorylation of Rb.

Inactivation of the genes composing the INK4 family of cell-cycle inhibitors is a frequent phenomenon in human cancer, although it seems to be mostly restricted to $p16^{\text{INK4a}}$ and $p15^{\text{INK4b}}$ silencing through genetic and epigenetic mechanisms. Alterations of both $p16^{\text{INK4a}}$ and $p15^{\text{INK4b}}$ have been described in non-Hodgkin lymphoma (NHL) and Hodgkin lymphoma (HL). In these hematological neoplasias, hypermethylation of the 5' CpG island of these genes seems to be a frequent event, whereas the incidence of homozygous deletion and mutations is relatively low. Unsurprisingly, this hypermethylation status of the promoter region correlates with transcriptional repression of these genes and constitutes an alternative inactivating mechanism.

The human $p18^{\text{INK4c}}$ gene was first identified in a yeast interaction screen that searched for CDK6-interacting proteins. $p18^{\text{INK4c}}$ was demonstrated to interact with CDK6 and more weakly with CDK4 (but not with other CDKs) both in vivo and in vitro, and to inhibit the kinase activity of cyclin D-CDK6 complexes. Ectopic expression of $p18^{\text{INK4c}}$ was shown to suppress cell growth in a wild-type Rb-dependent manner. $p18^{\text{INK4c}}$ has been proposed to function as a tumor suppressor gene, based on the observation that $p18^{\text{INK4c}}$-null mice display an increased susceptibility to developing spontaneous and induced tumors, such as pituitary adenomas and others. However, alterations in the $p18^{\text{INK4c}}$ gene are strikingly less frequent than those affecting $p16^{\text{INK4a}}$ or $p15^{\text{INK4b}}$, having been observed in human cancer only sporadically.

Although in normal lymphoid B-cells the $p18^{\text{INK4c}}$ protein has been implicated in key functions such as cell cycle control and terminal (plasma cell) differentiation, most studies performed in lymphoid neoplasms have shown preservation of the gene and its expression, except for myeloma-derived cell lines and, more rarely, tumors, where occasional homozygous deletions have been demonstrated. These findings could be considered unexpected due to the structural and functional homology of $p18^{\text{INK4c}}$ to $p16^{\text{INK4a}}$ and $p15^{\text{INK4b}}$, and its key role in cell
cycle control. Nevertheless, all these previous analyses largely ignored HL and HL-derived cell lines.

As the neoplastic Reed-Sternberg (RS) cells in HL constitute a paradigm of cell cycle arrest resistance\(^{25}\) and defective B-cell terminal differentiation\(^{26,27}\), we hypothesized that inactivation of the p18\(^{\text{INK4c}}\) gene might contribute to the disruption of the cell cycle control machinery in this neoplasia. Using a tissue microarray (TMA) approach, we examined p18\(^{\text{INK4c}}\) protein expression in normal lymphoid tissue and in a group of common B-cell lymphomas including follicular center (FCL), mantle cell (MCL), diffuse large B-cell (DLBCL), and Burkitt (BL) lymphomas, and compared the findings with a series of 316 HL tumors. Our results show loss of p18\(^{\text{INK4c}}\) protein expression in 45.3% of HL tumors compared with a normal expression pattern in the majority of NHLs. Furthermore, this loss of protein expression is frequently related with a hypermethylated status of the promoter region of the p18\(^{\text{INK4c}}\) gene in both HL-derived cell lines and tumors, as demonstrated by methylation-specific PCR (MSP) and bisulfite sequencing. Finally, loss of p18\(^{\text{INK4c}}\) protein in the RS cells of some HL tumors is associated with an unfavorable treatment response and a worse clinical outcome, underlining the biological and clinical relevance of this phenomenon.

**MATERIALS AND METHODS**

**Tumor samples and cell lines**

316 retrospective cases of HL were collected by collaborating members of the Spanish Hodgkin Lymphoma Study Group\(^{25}\). The histological confirmation of HL and subtype was determined by central review using standard tissue sections, and diagnoses were made according to the criteria of the WHO classification\(^{28}\). Cases included 171 cases of nodular sclerosis HL, 112 cases of mixed-cellularity HL, 14 cases of lymphocyte-rich classical HL, 9 cases of lymphocyte-depletion HL, and 10 cases of nodular lymphocyte-predominant HL. All the samples included represent at-diagnosis biopsies and all the patients were treated following standard protocols: patients with advanced HL were mainly treated with 6 to 10 courses of combination chemotherapy (ABVD or variants), whereas low-risk patients received extended-field radiotherapy or 2 to 4 courses of chemotherapy plus involved-field radiotherapy.

Paraffin-embedded blocks from reactive lymphoid tissue (reactive lymph nodes and tonsils) and 20 different NHL samples (including 6 FCL, 4 MCL, 6 DLBCL, and 4 BL) were obtained from the tissue archives of the CNIO Tumor Bank. These samples were included as internal controls in all the TMAs.

We obtained four HL-derived cell lines (L-540, HDLM-2, KM-H2 and L-428) and 7 NHL-derived cell lines (RAJI and NAMALWA, Burkitt lymphoma; GRANTA-519, mantle cell lymphoma; WSU-
NHL, KARPAS-422 and DOHH-2, diffuse large B-cell lymphomas with t(14;18); RPMI-8226, multiple myeloma) from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweigh, Germany). All cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10-20% fetal calf serum (GIBCO), glutamine, penicillin, and streptomycin.

**TMA design and immunohistochemistry (IHC)**

We used a Tissue Arrayer device (Beecher Instrument, MD) to construct the TMAs as previously described. Two selected 1-mm-diameter cylinders from two different areas were included in each case, along with the different controls to ensure the quality, reproducibility and homogenous staining of the slides. The robustness and reproducibility of this technique for analyzing HL has been previously demonstrated.

TMA blocks were sectioned at a thickness of 3 µm and dried for 16 hours at 56°C before being dewaxed in xylene, rehydrated through a graded ethanol series and washed with phosphate-buffered saline (PBS). Antigen retrieval was achieved by heat treatment in a pressure-cooker for 2 minutes in 10 mM citrate buffer (pH 6.5). Endogenous peroxidase was blocked and immunohistochemical staining was performed on these sections using a monoclonal anti-p18INK4c antibody (118.2, sc-9965, Santa Cruz Biotechnology, Santa Cruz, CA). Immunodetection was performed with the LSAB visualization System (DAKO, Glostrup, Denmark) employing diaminobenzidine chromogen as substrate. Sections were counterstained with hematoxylin.

The pattern of staining was recorded as positive or negative, depending on the expression in RS cells. Any degree of nuclear expression was considered as positive staining, since all p18INK4c-positive cases showed a significant amount of positive RS cells, although the intensity was variable from case to case. We considered as negative cases those without any noticeable p18INK4c nuclear expression in the tumoral cells. Reactive benign lymphocytes and plasma cells served as internal controls of the technique.

IHC techniques for p18INK4c protein expression were also performed on cytospin preparations of the different HL-derived cell lines fixed in ethanol/acetone (v/v), using the same procedures described for tissue sections.

**Western blot**

For extraction of total protein, cells were lysed in a buffer containing 10 mM Tris-HCl pH 7.4, 130 mM NaCl, 1% Triton X-100, plus protease and phosphatase inhibitors. Lysates were incubated on ice for 20 minutes and cleared by centrifugation. Approximately 80 µg of protein were electrophoresed in 12% SDS-PAGE gels and transferred overnight onto nitrocellulose
membranes. Membranes were sequentially incubated with blocking solution (5% nonfat milk in PBS - 0.1%Tween 20), the appropriate dilution of the primary antibody (1:100 for p18\textsuperscript{INK4c} and 1:10000 for α-tubulin) and horseradish peroxidase-conjugated secondary antibody (DAKO). Finally, the blots were developed using the enhanced chemiluminescence system (Amersham Biosciences, Freiburg, Germany). The antibodies used were anti-p18\textsuperscript{INK4c} (Santa Cruz Biotechnology) and anti-α-tubulin (Sigma, St. Louis, MO) as a loading control.

**Bisulfite treatment of DNA and methylation-specific PCR (MSP)**

MSP is based on the selective conversion of cytosine, but not methylcytosine, to uracyl by sodium bisulfite, followed by PCR amplification using specific primers that allow the discrimination between methylated and unmethylated modified sequences\textsuperscript{32}.

DNA was extracted from 26 randomly selected HL cases based on the availability of frozen tissue, and from all the cell lines, using standard phenol-chloroform methods. After extraction, 1 µg of DNA was denatured in 3 M NaOH at 42°C for 20 minutes and treated with 520 µl of 4.3 M sodium bisulfite, pH 5.0, in the presence of 30 µl of 20 mM hydroquinone at 50°C for 17 hours. DNA was then purified using the Wizard DNA Clean-Up system (Promega, Madison, WI), treated with 3 M NaOH at 37°C for 20 minutes, precipitated with ammonium acetate and ethanol and resuspended in 40 µl H₂O. As an initial positive control, in vitro methylated DNA was generated by treatment of 5 µg genomic DNA with 20 U SssI-methylase (New England Biolabs) and 160 µM S-adenosyl-methionine for 4 hours at 37°C.

Primer sequences for the methylated-specific reaction were 5′-GAT TTC GCG GGG TCG AAT TTC G-3′ (sense) and 5′-ACT AAC GCT CGC GCT CGC AA-3′ (antisense), whereas primer sequences for the unmethylated-specific reaction were 5′-GTT GGT AGG AGG TTG GTG TG-3′ (sense) and 5′-CAC CCT CCA CCC TAC TAA CAC TCA CA-3′ (antisense). These primer sets were designed according to previously described criteria\textsuperscript{32} to amplify 165 and 132 base pairs (bp), respectively, from the region immediately upstream of the first transcription start site, coincidental with a peak of maximum density of CpG sites.

The PCR reactions were performed in a total volume of 25 µl. Reaction mixtures contained 1X FastStart PCR buffer, 2 mM MgCl\textsubscript{2}, 0.2 mM of deoxynucleotide triphosphates (dNTPs), 10 pmol of each primer, 1.5 U of FastStart Taq polymerase (Roche), and 75 ng of bisulfite-modified DNA. PCR conditions were as follows: 5 minutes at 95°C, 40-42 cycles of amplification (30 seconds at 95°C, 30 seconds at 61°C, 30 seconds at 72°C), and 10 minutes at 72°C. All reactions were performed with positive and negative controls for both unmethylated and methylated alleles and a no DNA control. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.
Positive results for the MSP reactions were considered in those cell lines or HL cases showing detectable bands in the methylated-specific reaction (M) in at least two independent experiments.

**Bisulfite genomic sequencing**

In the bisulfite sequencing technique, the fragment of interest is amplified from bisulfite-modified DNA, cloned and sequenced, in order to obtain an accurate map of the distribution of CpG methylation.

A new primer set [5'-TAG GAA TTG GGG TAG TTG GGG-3' (sense) and 5'-TTT CCT TCA CTC CCT CCC TTA CTA C-3' (antisense)] was designed to amplify a region of 483 bp surrounding the first transcription start site that contained 44 CpG dinucleotides. PCR conditions were: 5 minutes at 95ºC, 42 cycles of amplification (30 seconds at 95ºC, 30 seconds at 58ºC, 75 seconds at 72ºC), and finally 10 minutes at 72ºC. The PCR mixture contained 1X FastStart PCR buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 20 pmol of each primer, 2 U of FastStart Taq polymerase, and 100 ng of bisulfite modified DNA in a final volume of 50 µl. PCR products were gel purified (QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany) and cloned into the pCR2.1-TOPO vector (TOPO-TA Cloning Kit, Invitrogen, Karlsruhe, Germany). Clones containing the desired insert were sequenced in an ABI 3700 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany).

**Laser capture microdissection and bisulfite sequencing of RS cells**

Laser capture microdissection (LCM) was performed to isolate single cells using a SL-Microtest instrument (MMI, Glattbrugg Switzerland). Pools of RS cells and normal reactive cells were dissected from 10-µm frozen tissue sections immunostained with a commercial anti-CD30 antibody (Novocastra, Newcastle-upon-Tyne, UK). Approximately 100 RS (large atypical CD30+) cells and 200-300 normal (CD30-) cells were isolated in separate vials. DNA was extracted by overnight digestion with proteinase K at 55ºC and ethanol precipitation, and a modified bisulfite treatment was performed according to Millar et al.

For genomic sequencing on microdissected cells, a 165 bp fragment containing 14 CpG dinucleotides was amplified using a seminested PCR. Primers sequences were as follows: 5'-TAG GAA TTG GGG TAG TTG GGG-3' (sense); 5'-CCC TCA TTC CC(G/A) CCT CC-3' (external antisense); 5'-TCC C(G/A)C TCT CCA CCT C-3' (internal antisense). PCR conditions were as previously published with slight modifications. The PCR products were gel-purified, cloned, and sequenced as described above.
Statistical study

For the purpose of statistical analysis, the clinical status of the different patients at diagnosis was defined using the Ann Arbor stage and the International Prognostic Score (IPS). The Pearson chi-square test was used where appropriate to establish whether there were any relationships between the clinical characteristics of the patients and IHC results.

Survival analyses were performed on all the classical HL cases for which clinical and follow-up information was available (approximately 70%). Actuarial survival curves, in terms of overall survival (OS), were plotted using the Kaplan and Meier method. Statistical significance of associations between individual variables and OS was determined by using the log-rank test. A multivariate Cox model was used to compare the predictive performance with the IPS prediction. All statistical analyses addressing the clinical outcome of the patients were restricted to HIV-negative patients with classical HL.

All tests were two-sided and P values of 0.05 or less were considered statistically significant. The SPSS (SPSS Inc. Chicago IL: SPSS; 1999) software package was used for these analyses.

RESULTS

The pattern of expression of p18\textsuperscript{INK4c} protein is abnormal in HL tumor samples, as compared with normal lymphoid tissue.

All the samples from reactive lymphoid tissue tested by IHC displayed nuclear (and, to a lesser extent, cytoplasmic) staining by most germinal center lymphocytes (more intensely in mature centrocytes and plasma cells), whereas mantle zone lymphocytes were usually unstained (figure 1A). The large majority of mature plasma cells in the interfollicular areas were also positive. These differences are consistent with the proposed regulation of p18\textsuperscript{INK4c} protein expression through normal B-cell differentiation.

NHLs recapitulated the differences observed in p18\textsuperscript{INK4c} protein expression in reactive lymphoid tissue: tumors derived from follicular center cells (all the FCL, DLBCL, and BL samples included in the TMAs: 16/20) expressed nuclear reactivity for p18\textsuperscript{INK4c}, whereas all MCL tumors were negative (data not shown).

In contrast, a substantial number of HL samples showed no p18\textsuperscript{INK4c} nuclear staining in the RS cells (143/316 samples, 45.3%) (Figure 1A). Samples recorded as p18\textsuperscript{INK4c}-positive always showed a significant amount of positive RS cells, this contrasting with the complete absence of
tumoral cells with p18\(^{\text{INK4c}}\) nuclear expression in cases considered as negative. The loss of p18\(^{\text{INK4c}}\) nuclear expression in the tumoral cells was found in both forms, classical HL (136/306) and nodular lymphocyte-predominant HL (7/10 cases). This observation suggests an abnormal pattern of p18\(^{\text{INK4c}}\) protein expression, apparently restricted to HL tumors.

**p18\(^{\text{INK4c}}\) gene promoter hypermethylation in HL-derived cell lines**

The genomic region containing the promoter and first exon of the p18\(^{\text{INK4c}}\) gene displays a relatively high density of CpG dinucleotides. Since the commonest mechanism of CKI loss in hematological malignancies is promoter hypermethylation, we investigated by MSP the methylation status of the p18\(^{\text{INK4c}}\) gene in the HL cell lines, and compared it to that of NHL cell lines. We found that KM-H2 and L-428 only displayed amplification bands for the unmethylated reaction, whereas L-540 and HDLM-2 amplified in both the M and U reactions (figure 1B). Six of 7 NHL-derived cell lines (RAJI, NAMALWA, GRANTA-519, WSU-NHL, KARPAS-422, DOHH-2) only showed unmethylated bands in the MSP assays (figure 1B). The multiple myeloma-derived cell line RPMI-8226 failed to amplify either M or U bands, a finding consistent with the previously described sporadic homozygous deletion of the p18\(^{\text{INK4c}}\) gene in myeloma cell lines\(^24\). The existence of this deletion in RPMI-8226, but not in any of the other cell lines, was confirmed by multiplex PCR using unmodified genomic DNA as a template (data not shown).

As might be expected, this hypermethylated status of the p18\(^{\text{INK4c}}\) promoter correlated with loss of protein expression as demonstrated by Western blot and IHC (figure 1C). p18\(^{\text{INK4c}}\) protein expression was almost completely absent from the L-540 cell line, and clearly reduced in HDLM-2 and KM-H2 compared with the L-428 cell line.

The amplification of U and M bands in some HL-derived cell lines probably reflects inter-allelic heterogeneity of CpG island methylation. To confirm the methylation-associated silencing of the p18\(^{\text{INK4c}}\) gene, and to analyze the pattern of CpG island methylation, we performed bisulfite genomic sequencing of a fragment of the promoter region of p18\(^{\text{INK4c}}\) containing 44 CpG sites (as indicated in figure 2A) in the L-540 and HDLM-2 cell lines. The L-540 cell line showed a high degree of CpG methylation in most sequenced clones, whereas only 2 clones from HDLM-2 were hypermethylated. These results are consistent with the results obtained by MSP, Western-blot and IHC.
Figure 1: Analysis of p18\textsuperscript{INK4c} protein expression and promoter hypermethylation. (A) Immunohistochemical detection of p18\textsuperscript{INK4c} protein. In reactive lymphoid tissue (left panel: a benign lymph node), p18\textsuperscript{INK4c} is expressed mainly by lymphocytes in germinal centers and interfollicular plasma cells, but not by mantle cells. Middle and right panels: examples of HL cases showing absence and presence of p18\textsuperscript{INK4c} protein expression by RS cells, respectively. (B) MSP analysis of the methylation status of the p18\textsuperscript{INK4c} promoter in HL-derived cell lines (top), NHL-derived cell lines (middle) and HL tumors (bottom). IVMD, in vitro-methylated DNA; NS, DNA from a sample of normal spleen, used as a negative (unmethylated) control; U, unmethylated; M, methylated. (C) p18\textsuperscript{INK4c} protein expression in HL-derived cell lines. Top: Western blot analysis of p18\textsuperscript{INK4c} expression in total protein extracts from the HL cell lines. The multiple myeloma cell line RPMI-8226 shows total absence of p18\textsuperscript{INK4c} expression and was included as a negative control. The expression of α-tubulin was analyzed as a loading control. Bottom: Immunohistochemical staining for p18\textsuperscript{INK4c} on cytospin preparations of the HL-derived cell lines.
The expression of \( \text{p18}^{\text{INK4c}} \) protein in RS cells in primary tumors is also abolished by gene promoter hypermethylation

The results obtained in cell lines suggest that this epigenetic silencing of \( \text{p18}^{\text{INK4c}} \) expression may also occur in primary tumors. To determine if the MSP assay was suitable for the detection of minoritary amounts of methylated DNA, such as those probably present in HL samples, a sensitivity study was performed. We were able to consistently detect methylated \( \text{p18}^{\text{INK4c}} \) alleles in dilutions up to 1:1000 (0.1%) of the methylated L-540 cell line in unmethylated DNA (L-428) (data not shown).

We analyzed the methylation status of the \( \text{p18}^{\text{INK4c}} \) gene by MSP in DNA extracted from HL affected tissues. \( \text{p18}^{\text{INK4c}} \) hypermethylation was detected in 19.2% (5/26 of tumoral samples) (figure 1B). As might be expected, all samples also had unmethylated alleles, presumably corresponding to surrounding non-malignant cells.

Since the loss of \( \text{p18}^{\text{INK4c}} \) protein expression detected by IHC is restricted to the RS cells, we speculated that hypermethylation could most likely be attributed to this tumoral component. To demonstrate that the positive signal in the MSP assay originated from RS cells, we microdissected RS and benign cells from one of the MSP-positive HL cases and performed bisulfite sequencing on DNA isolated from the 2 separate cell populations (figure 2B). High-density methylation of the analyzed sequence was only detected in DNA from RS cells although, as in the cell lines, inter-allelic heterogeneity was observed. None of the analyzed clones from microdissected reactive cells contained hypermethylated DNA.

The comparison with the results for \( \text{p18}^{\text{INK4c}} \) protein expression showed that all hypermethylated cases were negative by IHC (5/5). Cases showing only amplification of U bands by MSP expressed \( \text{p18}^{\text{INK4c}} \) protein in a variable proportion of cases: 9/16 cases were \( \text{p18}^{\text{INK4c}} \)-positive and 7/16 were negative. These results probably reflect the existence of alternative inactivation mechanisms, but we cannot exclude a number of false negative results in some HL samples due to the scarcity of RS cells.
The loss of p18\textsuperscript{INK4c} protein expression in HL tumors is associated with less favorable clinical outcome

To test the clinical relevance of the loss of p18\textsuperscript{INK4c}, we compared some clinical features (stage and IPS) at presentation, and follow-up of the HIV-negative, classical HL patients. p18\textsuperscript{INK4c} loss was independent of the clinical presentation and stage at diagnosis, but there was a clear relationship with treatment response, since 61% of p18\textsuperscript{INK4c}-negative cases had a bad treatment response compared with 39% of p18\textsuperscript{INK4c}-positive cases (treatment response was considered unfavorable in patients who either did not achieve complete remission after treatment or had...
relapsed less than 12 months after remission), although the difference just failed to meet the criterion for significance (p=0.055).

In agreement with this finding, the two groups of p18\textsuperscript{INK4c}-positive and p18\textsuperscript{INK4c}-negative cases had significant differences in the overall survival probability, as indicated by univariate analyses, during the follow-up period (log-rank=4.45, p=0.0349), with the p18\textsuperscript{INK4c}-negative phenotype being associated with shorter survival times. Survival curves for the two groups are shown in figure 3.

A multivariate Cox model including p18\textsuperscript{INK4c} protein expression in HL tumors and the IPS was fitted (table 1). The hazard ratios from the multivariate analysis were both significant, thus showing that the prognostic impact of the p18\textsuperscript{INK4c} loss can be regarded as independent of the conventional IPS prognostic system.

![Figure 3: Kaplan-Meier analysis of overall survival in HL patients grouped according to the expression of p18\textsuperscript{INK4c}.](image)

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\(\chi^2 = 24.376; p < 0.001\)

Table 1: Multivariate Cox model including p18\textsuperscript{INK4c} protein expression and IPS. Abbreviations: B, estimated coefficients; SE(B), standard errors; HR, hazard ratios with 95% confidence intervals (CI); p, p values corresponding to Wald’s statistic for each coefficient.
DISCUSSION

HL is a distinct primary lymphoid malignancy, classically associated with an abundance of inflammatory cells that outnumber the recognized tumoral subpopulation of RS cells. Defects in the regulation of the cell cycle, apoptosis, and signaling pathways have been repeatedly demonstrated in these cells. Additionally, the RS cells harbor clonally rearranged and somatically mutated immunoglobulin genes, indicating that they derive from germinal center B-cells, although they characteristically show a defective B-cell terminal differentiation program.

Loss of different CKIs has been demonstrated in HL tumors. Thus, hypermethylation of the 5’ CpG island of the p16\(^{INK4a}\) and p15\(^{INK4b}\) promoters associated with loss of protein expression seems to constitute a relatively frequent event. In the majority of cases, p27\(^{KIP1}\) protein is also lost, probably as a consequence of increased degradation mediated by SKP2, a ubiquitin ligase for p27\(^{KIP1}\) that has been found overexpressed in RS cells in most HL cases. Absence of p21\(^{WAF1}\) protein expression is detected only in a fraction of cases.

In addition to these well characterized alterations in this tumoral model, here we demonstrate a p18\(^{INK4c}\) transcriptional repression associated with promoter hypermethylation in some HL-derived cell lines and primary tumors. This finding in human tumors is comparable to previous observations in mouse models, suggesting that p18\(^{INK4c}\) functions as a tumor suppressor gene. Although pituitary tumors are the main finding in p18\(^{INK4c}\)-null mice, a higher proliferative rate upon mitogenic stimulation of B- and T-lymphocytes, and the development of lymphoproliferative disorders including B-cell and T-cell lymphomas have also been demonstrated in these models.

In addition to the frequent loss of CKIs, among them p18\(^{INK4c}\), the tumoral RS cells are also characterized by the increased expression in a large proportion of cases of cyclins and CDKs involved in G1/S and G2/M transitions, such as cyclin D, cyclin A, cyclin B1, cyclin E, CDK2 and CDK6, which differs from the observations in reactive lymphoid tissue and other NHLs. Since the activation of CDK6 may induce the transcription of p18\(^{INK4c}\) via E2F-1 activation, the simultaneous overexpression of CDK6 and p18\(^{INK4c}\) inactivation suggest that the loss of this autoregulatory mechanism may be important in these tumors.

Cell cycle arrest is tightly coupled to terminal differentiation of late-stage B-cells. Thus, p18\(^{INK4c}\) inhibition of CDK6 plays a pivotal role in integrating cell cycle arrest with terminal (plasma cell) differentiation. The p18\(^{INK4c}\) requirement in terminal B-cell differentiation is specific, since p18\(^{INK4c}\) deficiency cannot be compensated by other CKIs in vivo, including p19\(^{INK4d}\), p21\(^{WAF1}\), or p27\(^{KIP1}\). This link between cell cycle control and terminal differentiation may, at least in part, be related to the defective B-cell differentiation state characteristic of RS cells.
The pathogenic relevance of p18\textsuperscript{INK4c} in HL is additionally strengthened by the clearly established clinical relevance to the outcome of HL patients. HIV-negative, classical HL cases featuring loss of p18\textsuperscript{INK4c} protein expression behave significantly worse than p18\textsuperscript{INK4c}-positive cases, display poorer treatment response (lower probability of having sustained complete remission) and show inferior survival. Moreover, the loss of p18\textsuperscript{INK4c} is independent of the clinical stage or clinical extension of the tumors at diagnosis, and predicts survival independently of the IPS. It is noteworthy that previous reports failed to demonstrate clear relationships between inactivation of other CKIs and the clinical outcome in HL patients\textsuperscript{25}, highlighting the biological relevance of p18\textsuperscript{INK4c} in this specific tumor. However, further studies are needed to clarify these relationships and the exact role of p18\textsuperscript{INK4c}-silencing in HL and other tumors, and to investigate the CKI alterations present in p18\textsuperscript{INK4c}-positive cases.

In summary, our results demonstrate a common p18\textsuperscript{INK4c} transcriptional repression due to promoter hypermethylation in HL tumors and HL-derived cell lines. This is the first report of p18\textsuperscript{INK4c} silencing in human tumors due to epigenetic mechanisms, adding p18\textsuperscript{INK4c} to the long list of tumor suppressor genes whose expression can be silenced through promoter methylation. The combination of this and other defects in the cell cycle could not only be related with abnormal cell cycle regulation, but it also seems to couple the abnormalities in cell cycle with defective B-cell differentiation in RS cells.

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Silencing of the \( p18^{\text{INK4c}} \) gene by promoter hypermethylation in the Reed-Sternberg cells in Hodgkin Lymphomas

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