Cellular Senescence Markers p16\textsuperscript{INK4a} and p21\textsuperscript{CIP1/WAF} Are Predictors of Hodgkin Lymphoma Outcome

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Abstract

Purpose: There is evidence that Hodgkin Reed-Sternberg (HRS) cells in classical Hodgkin lymphoma (cHL) could display some molecular and morphologic markers of cellular senescence (CS). We hypothesized that CS mechanisms may have potential prognostic relevance in cHL and investigated whether the expression of the well-established CS biomarkers p21\textsuperscript{CIP1/WAF} and p16\textsuperscript{INK4a} by HRS cells might be predictive of the probability of event-free survival (EFS).

Experimental Design: The study analyzed a retrospective cohort of 147 patients and the results were validated on a cohort of 91 patients independently diagnosed and treated in a different institution. p16\textsuperscript{INK4a} and p21\textsuperscript{CIP1/WAF} were categorized as dichotomous variables (< or ≥ 30% of HRS cells at diagnosis) and evaluated in univariate and multivariate analysis.

Introduction

Classical Hodgkin lymphoma (cHL) has an incidence of about 3 cases per 100,000 people per year and is, therefore, quite frequent among lymphomas. To date, available treatments for cHL achieve cure rates of 70% and 80%, based on standard chemotherapy refined in some instances by radiotherapy (1). However, nonresponder and early relapsed patients still represent a therapeutic challenge, and their prognosis remains poor despite incremental treatment intensification (2). Why the majority of cHL patients behave so well, and why a small subclass of cHL is refractory to treatment remain unanswered questions. Though some clinical and laboratory prognostic markers have been figured out and tested (for example, we have previously described sCD30 as a neoplastic mass index and a reliable prognostic marker; refs. 3, 4), it is quite surprising that so far there is no acknowledged biologic feature linked to the degree of malignant behavior of cHL (5–7). Indeed, the pathogenesis of cHL has been intensively investigated, revealing a biologic heterogeneity that might provide useful information for clinical and prognostic case stratification. cHL is a lymphoma derived from B cells (8), characterized by rare tumor cells, the Hodgkin and Reed-Sternberg (HRS) cells, surrounded by an abundant reactive microenvironment. The majority of cells in affected tissues of cHL are a mixed infiltrate of different cell types, including macrophages, T and B lymphocytes, plasma cells, neutrophils, eosinophils, and mast cells. Experimental and in situ observations suggest that HRS cells secrete a variety of cytokines, chemokines, and cellular messengers (9, 10) and actively attract many of the above cells.

Despite the clinical and morphologic heterogeneity of cHL, only two signaling pathways, JAK-STAT and NF-kB, essentially drive its pathogenesis (10). In cHL, the constitutive activation...
of NF-κB and JAK-STAT can be caused by multiple genetic lesions interfering with these pathways, including genomic gains of NF-κB activators such as REL and NIK, inactivating mutations of NF-κB inhibitors such as IkBα and IkBε, and other lesions causing JAK–STAT pathway dysregulation (10). In addition, dysregulation of both classical and alternative NF-κB pathways can be ascribed to the expression of viral transforming proteins (e.g., LMP1) in EBV-infected HRS in a large number (nearly 40%) of cHL patients (10). Several studies conducted by us and others have demonstrated that some genes involved in the cell-cycle control are dysregulated in cHL (11–14). Among them are the cyclin-dependent kinase inhibitors p21CIP1/WAF1 and p16INK4a, which are the important components of two major tumor-suppressor pathways, p53 and Rb (15). Interestingly, these inhibitors are among the main drivers of the irreversible and permanent cell-cycle arrest observed in cellular senescence (CS), a protective mechanism against unlimited cell proliferation that could be induced by various stressful stimuli, including oxidative damage, telomere dysfunction, DNA damage, and aberrant oncogene activation (16). Moreover, it has been demonstrated that senescent cells may acquire a senescence-associated secretory phenotype (SASP), defined as an exaggerated production of proinflammatory signals (17, 18). Of note, SASP is dependent on the activation of the NF-κB pathway (19), and in this manner, senescent cells are able to alter the tissue microenvironment where they home. Another important point is that cytotoxic effects of various chemotherapeutic drugs and ionizing radiation are also accompanied by the induction of CS (20, 21). Finally, there are multiple molecular links between CS and cancer, including common genes, microRNAs, and signaling pathways (22). Considering the above, it is plausible to hypothesize that the mechanisms of CS are involved in cHL, with potential prognostic relevance. We further suggest that a good prognosis is associated with the ability of HRS cells to acquire a CS-like phenotype, whereas a subgroup of cHL, which contains senescence-escaping cells that are more resistant to conventional therapies, is associated with a bad prognosis.

To address these hypotheses, we analyzed HRS cells for the expression of well-established CS biomarkers, including p21CIP1/WAF1 and p16INK4a. Furthermore, in a large retrospective series of cHL cases, we investigated whether the presence of p21CIP1/WAF1 and p16INK4a might be predictive of the response to treatment in patients with cHL.

Materials and Methods

Patients and samples

A retrospective study was performed in a primary cohort of patients with cHL who underwent treatment between 1986 and 2012 (n = 147). These cases were collected from the archives of the Pathology Department of Verona University. A validation cohort of cHL patients (n = 91) diagnosed between 1991 and 2011 was collected from the archives of the Pathology Department of the Vita Salute University in Milan. Case selection was based on the availability of paraffin-embedded formalin-fixed lymph node from the initial diagnosis in untreated patients and of clinical data. All samples were reviewed independently by two expert hematopathologists (M. Chilosi and A. Zamò). Diagnosis of cHL was established on the basis of the WHO 2008 criteria (23). Written informed consent was signed by the patients; when not possible (dead or unreachable patients), the study material was used after deidentification according to the Italian law and with the approval of the institutional ethic committee. All procedures were done in accordance with the Helsinki declaration.

Clinical staging and treatment

Patients were staged according to Ann Arbor criteria (24). Staging procedures included clinical examination, blood tests, chest X-rays, chest/abdomen CT, abdominal ultrasonography, and bone marrow biopsy. More recent patients were also staged by PET-CT. Bulky disease was defined as mediastinal mass exceeding one third of the thoracic diameter measured at the D5-D6 level and/or extramediastinal mass ≥10 cm. Unfavorable stages included bulky disease and III/IV stages. Patients received stage-directed treatment based on 4 to 6 courses of ABVD chemotherapy with or without field radiotherapy (25, 26). Complete remission (CR) was defined as the complete disappearance of clinical, physical, radiologic, and biochemical abnormalities due to cHL. Partial remission (PR) was defined as the reduction of at least 50% for at least 4 weeks of all measurable masses with no new lesions appearing and no progression at the original sites of the disease or progression (increase of any measured lesion or the appearance of new lesions) was considered as treatment failure. Clinical assessment after treatment was performed every 3 or 4 months for the first year, every 6 months for the second and third years and once a year thereafter.

Immunohistochemical staining

All tissue samples were formalin-fixed and paraffin-embedded according to standard methods. Sections from tissue blocks of cHL were immunohistochemically analyzed with the following primary antibodies: CD30 (clone BerH2; Dako; dilution 1:50); OCT2 (rabbit polyclonal; Santa Cruz Biotechnology; dilution 1:200); BOB1 (clone C-20; Santa Cruz Biotechnology; dilution 1:1,500); PAX5 (clone IEW; Novocastra; dilution 1:50); CD15 (clone Carb-3; Dako; dilution 1:30); p21CIP1/WAF1 (clone SX 118; Dako; dilution 1:50); p16INK4a (clone IC 8; Santa Cruz Biotechnology; dilution 1:50). All cases were tested for the presence of EBV by LMP1 immunostaining (clone CS 1-4; Dako; dilution 1:200) and using in situ
hybridization for Epstein-Barr virus early RNAs (EBER; NCL-E BV; Novocastra). All staining procedures were performed in an immunostainer (Bond-Max, Leica).

HRS cells were considered positive for p21\(^{\text{CIP1/WAF1}}\) when a distinct nuclear staining could be shown, whereas the cells were considered as positive for p16\(^{\text{INK4a}}\) when either the cytoplasm or the nucleus or both were stained. Ten reactive lymphoid tissues with an abundant reactive follicular component were selected as controls, including 7 lymph nodes and 3 tonsils. In control samples, low levels of p16\(^{\text{INK4a}}\) cytoplasmatic immunoreactivity were observed in dendritic cells and also in epithelial cells in reactive tonsils; p21\(^{\text{CIP1/WAF1}}\) expression was found in scattered nuclei in reactive follicular component and parabasal epithelial cells in reactive tonsils.

The quantitative evaluation of p16\(^{\text{INK4a}}\) and p21\(^{\text{CIP1/WAF1}}\) in HRS was performed on images of selected fields captured with a scanning microscopy device (D-Sight). The score of p16\(^{\text{INK4a}}\), and p21\(^{\text{CIP1/WAF1}}\) reactive HRS cells was obtained by evaluating the percentage of positive atypical cells, as well as by comparison with CD30 immunostaining on serial sections. According to the percentage of positive HRS cells, four scores were defined: negative (0), low (1), moderate (2), and high (3). The assigned scores were as follows: score 0, devoid of expression or in less than 10% positive HRS cells; score 1, 10% to 29% positive HRS cells; score 2, 30% to 59% of positive HRS cells; score 3, ≥60% of positive HRS cells (27). The concordance rate between the two pathologists was 88% for p16 and 92% for p21. Discordant cases were reviewed at a multihead microscope and a consensus was reached for each case.

The consistency of score assignment was confirmed on selected cases using double immunostaining with CD30 and either p16\(^{\text{INK4a}}\) or p21\(^{\text{CIP1/WAF1}}\). This four-tiered scoring system was easily reproducible (27). For statistical purposes, it was transformed in a two-tiered system by considering a 30% cutoff level.

Bioinformatic analysis

We used the MARQ online tool (http://MARQ.dacya.ucm.es; ref. 28) for searching gene-expression signatures similar to those formed in a two-tiered system by considering a 30% cutoff level. For statistical purposes, it was transformed in a two-tiered system by considering a 30% cutoff level.

Statistical analysis

Data were analyzed using the statistical software Stata 12.1 (www.stata.com). Continuous variables were summarized as the median and range or the mean ± SEM. Data were summarized by percentages for categorical variables and by medians and ranges for survival times. Main analyses assumed as end-point the event-free survival (EFS), defined as the time interval between the beginning of treatment to primary treatment failure, relapse or death from any cause or last follow-up. EFS curves were estimated by the Kaplan–Meier method (30). In univariate analysis, we tested the following variables by log-rank test or, for continuous variables, Cox’s proportional hazard regression model (31, 32): p21\(^{\text{CIP1/WAF1}}\) and p16\(^{\text{INK4a}}\), EBER, LMP1, CD30 (absent or low expression vs. high expression for each molecule), favorable versus unfavorable stage, histotype, sex and, as continuous variables, age, Hb, LDH, albumin, fibrinogen, and ESR. To analyze EFS in multivariate analysis by the Cox’s proportional hazards model, the independent variables were chosen on the basis of their significance in univariate analysis and/or of their recognized relevance to outcome in cHL. We examined the predictive ability of the model by applying the Harrell c-statistic (33); a c-statistic near 1 or near 0 indicates greater predictive ability. p21\(^{\text{CIP1/WAF1}}\)/p16\(^{\text{INK4a}}\) were also considered together as a categorical variable (both <30%, either <30%, both ≥30%) and compared by the log-rank test. In Supplementary Data, the primary cohort was further studied, including sCD30 (< or ≥ 100 U/mL; available for only 132 patients in the primary cohort), into the multivariate analysis and comparing p21\(^{\text{CIP1/WAF1}}\)/p16\(^{\text{INK4a}}\) as a categorical variable within <100 and ≥100 U/mL sCD30 subgroups. Results were regarded as statistically significant for P < 0.05.

Results

Patient characteristics

The main clinicopathologic characteristics of the patients (Table 1) were similar between the primary and the validation cohort.
cohort. The two cohorts were dissimilar for patient number (147 vs. 91), median follow-up (11 vs. 5 years), bulky disease (42 vs. 16), and adverse event ratio (30 vs. 21%). In the primary cohort, the median follow-up time from the beginning of treatment was 11 years for surviving patients (range from 3 months to 26 years). A CR was obtained in 144 (98%) of 147 patients (3 progressed during treatment). Forty-one (28%) of 144 patients relapsed. As a whole, 103 (70%) of 147 patients persisted in CR and 44 (30%) experienced adverse events. At the end of the study, 23 (16%) of 147 patients had died: 22 as a direct consequence of the disease and one from apparently cHL-unrelated cause. In the validation cohort, the median follow-up time was 5 years (range from 1 month to 20 years). A CR was obtained in 88 (97%) of 91 patients (3 progressed during treatment). Sixteen (18%) of 89 patients relapsed. As a whole, 73 (80%) of 91 patients persisted in CR and 19 (21%) presented with adverse events. At the end of the study, 9 (10%) of 91 patients had died: 7 due to cHL and 2 from apparently unrelated causes.

Prognostic significance of HRS senescence markers in the primary cohort

The number of HRS cells expressing p16\(^{INK4a}\) and p21\(^{CIP1/WAF1}\) on paraffin-embedded lymph node samples varied within a wide range, from rare to the majority of cells. In 48% of the cases, over 30% of the HRS cells expressed p16\(^{INK4a}\) and p21\(^{CIP1/WAF1}\) (Fig. 1; Table 1).

Figure 1.
Expression of CS-related markers p16\(^{INK4a}\) and p21\(^{CIP1/WAF1}\) in HRS cells in cHL (H&E ×20, A and B). The quantitative scoring system used in this study is exemplified in a positive case (column C, E, and G) and a negative case (column, D, F, and H). CD30 (×20, C and D); p16\(^{INK4a}\) (×20, E and F); p21\(^{CIP1/WAF1}\) (×20, G and H).
Results from our univariate analysis indicated that p16INK4a and p21CIP1/WAF1 were powerful prognostic factors in terms of EFS probability. Their levels of expression at diagnosis in relation to clinical features are listed in Table 1 and their frequencies of expression were almost uniformly distributed. p16INK4a and p21CIP1/WAF1 were categorized as dichotomous variables (<30% or ≥30% of HRS cells at diagnosis) and Fig. 2A depicts the Kaplan–Meier plot of EFS accordingly. The difference between EFS curves was significant (P < 0.001) for both p16INK4a and p21CIP1/WAF1 (Fig. 2A). The EFS probability at the plateau was 45% (95% confidence interval (CI), 33–57) for lower versus 89% (95% CI, 77–95) for higher p16INK4a samples, and 40% (95% CI, 28–53) for lower versus 90% (95% CI, 79–95) for higher p21CIP1/WAF1 samples. p16INK4a/p21CIP1/WAF1 were also considered together as a unique categorical variable (both < 30%, either <30%, both ≥30%). This combined variable defined three different prognostic groups associated with statistically significant different EFS probabilities either overall or within each stage, the best prognosis being associated with the robust expression of both molecules (Figs. 2A and 3A; Table 3).

On the basis of our multivariate analysis (Table 2), both p16INK4a and p21CIP1/WAF1 were independent prognostic factors, with ≥30% expression being protective against relapse (HR, 0.25; 95% CI, 0.11–0.57; P = 0.001 and HR, 0.16; 95% CI, 0.06–0.37; P < 0.001, respectively). Bulky and III–IV stages were also confirmed to be independent prognostic factors in these patients (HR, 3.79; 95% CI, 1.54–9.29; P = 0.004 and HR, 2.25; 95% CI, 1.00–5.07; P = 0.051, respectively). The Harrell C-statistic was equal to 0.82, indicating a good predictive discrimination and the effectiveness of the Cox’s model. Further comparative analyses considering also sCD30 levels are available as Supplementary Data (Supplementary Fig. S1; Supplementary Tables S1 and S2).

Prognostic significance of HRS senescence markers in the validation cohort

The prognostic value of p16INK4a and p21CIP1/WAF1 expression in HRS cells was further confirmed in an independent validation cohort of 91 patients. The results were similar to the primary cohort (Fig. 2A). p16INK4a or p21CIP1/WAF1 expression in ≥ or <30% of HRS cells remained associated with different EFS
probabilities \( (P < 0.001) \). The combined variable \( p16^{INK4a} \) and \( p21^{CIP1/WAF1} \) entailed different, statistically significant EFS probabilities, associated with a robust or the lack of expression of the molecules. Here also, the best outcome was associated with a robust expression of both molecules either overall or within each stage (Figs. 2B and 3B; Table 3).

The multivariate analysis confirmed that \( p16^{INK4a} \) and \( p21^{CIP1/WAF1} \) were independent prognostic variables with higher levels conferring statistically significant protection against relapse (HR, 0.19; 95% CI, 0.05–0.69; \( P = 0.011 \) and HR, 0.25; 95% CI, 0.07–0.85; \( P = 0.026 \), respectively). The good predictive discrimination ability and the effectiveness of the Cox’s model were defined by a Harrell \( c \)-statistic of 0.82.

Similar gene signatures: MARQ-based analysis

As could be expected, the majority of gene-expression signatures similar to cHL include various types of cancer (Supplementary Table S3; ref. 7). With regard to our findings that \( p16^{INK4a} \) and \( p21^{CIP1/WAF1} \) are often expressed in cHL, we anticipated that some similarity should be observed with gene-expression profiles for CS. However, such a similarity was not found. This could be attributed to the fact that even for cHL with a good prognosis, only a small number of cells in the tumor expressed the CS markers. Nevertheless, an interesting observation was that the gene-expression profile of cHL with good prognosis was similar to the gene signature of miR-124 overexpression (similarity score of \( P < 0.0001 \)). This tumor-suppressor microRNA is implicated both in many types of cancer and in the CS network (22).

Table 2. Multivariate analysis for event occurrence

<table>
<thead>
<tr>
<th>Stage (vs. I-II)</th>
<th>Primary cohort HR (95% CI)</th>
<th>( P )</th>
<th>Validation cohort HR (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \geq 30% )</td>
<td>0.25 (0.11–0.57)</td>
<td>0.001</td>
<td>0.19 (0.05–0.69)</td>
<td>0.011</td>
</tr>
<tr>
<td>(&lt; 30% ) p16/21</td>
<td>0.16 (0.06–0.37)</td>
<td>&lt;0.001</td>
<td>0.25 (0.07–0.85)</td>
<td>0.026</td>
</tr>
<tr>
<td>CD30</td>
<td>0.90 (0.45–1.76)</td>
<td>0.752</td>
<td>1.56 (0.55–4.43)</td>
<td>0.408</td>
</tr>
<tr>
<td>Bulky</td>
<td>3.79 (1.54–9.29)</td>
<td>0.004</td>
<td>1.34 (0.39–5.55)</td>
<td>0.638</td>
</tr>
<tr>
<td>III–IV</td>
<td>2.25 (0.00–5.07)</td>
<td>0.001</td>
<td>1.81 (0.62–5.24)</td>
<td>0.277</td>
</tr>
<tr>
<td>Age*</td>
<td>1.20 (0.98–1.48)</td>
<td>0.075</td>
<td>1.13 (0.82–1.53)</td>
<td>0.449</td>
</tr>
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NOTE: Statistically significant \( P \) values are shown in bold type.

*HR calculated on the basis of an increase of 10 years.
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Discussion

The main hypothesis investigated in this article is that a subset of HRS cells display a CS-like phenotype, and that senescence-escaping mechanisms might make HRS cells more resistant to common therapeutic regimens, thus affecting patients' prognosis. Both hypotheses seem to be supported by our data, because we showed a consistent expression of CS-markers and may be useful for the detection of this response in vivo. According to our hypothesis, HRS cells exhibit a variety of features of senescent cells, including abnormal cell enlargement, nuclear disorganization with aberrant expression of cell-cycle regulatory factors, abnormal karyokinesis, and telomere aberrant distribution (38, 39). Also, the recruitment of inflammatory cells around HRS cells, a typical morphologic feature of cHL, can be ascribed in this scheme to the CS-associated cytokine storm" (SASP), a well-known feature characterizing experimentally induced CS (35).

Our study provides evidence that the quantitative evaluation of CS markers in HRS cells of cHL can have a prognostic significance in line with, and probably grounded on, their biologic role analogously to other neoplasias (40–42).

Previous studies described some relationship between the expression of cell-cycle protein and patient outcome. For example, in non-Hodgkin lymphoma, the levels of p53/p21CIP1/WAF1 (12, 43) and Rb/p16INK4a have been found to correlate with clinical progression and predict OS. Regarding cHL, Guenova and colleagues (13) reported high-frequency p16INK4a expression in all 44 cases of cHL, but they did not find any statistical prognostic correlation, probably due to the small number of cases and the shorter follow-up. Similarly, Garcia and colleagues (44, 45) reported the loss of p16INK4a expression in 73% and in 82.5% of cHL and a trend for association of a shorter OS with low p21CIP1/WAF1 expression was observed (44). In this study, we provide strong evidence that in cHL, an inverse relationship exists between the clinical outcome and the expression of p16INK4a and p21CIP1/WAF1, and this relationship is robust and easily detected on routine histologic preparations. The multivariate analysis on two different series from independent institutions shows that both of the CS markers retain independent prognostic significance among the parameters analyzed. The prognostic power may be further refined by considering p16INK4a/p21CIP1/WAF1 together as a unique categorical double-positive, double-negative, or either-negative variable. Thus categorized, it entails subpopulations with dramatically divergent EFS probabilities. As suggested by the biologic role of CS, it is the lack or the presence of the robust expression of both CDKIs that define the worst or the best prognosis in our series. This suggests that a worst prognosis subgroup of cHL patients could be sorted out to be treated differently. It also points to the potential of sensitizing CS-resistant tumors to CS induction by drugs in cHL treatment. In addition, we show (although only in the primary cohort) that the two CDKIs may be combined with the mass-dependent parameter sCD30 (3, 4) into an even more refined prognostic index (Supplementary Fig. S1; Supplementary Tables S1 and S2). The prognostic power of p16INK4a and p21CIP1/WAF1 could be variably explained. One of the possibilities is that the inactivation of the major tumor-suppressor pathways, such as p53/p21CIP1/WAF1 and pRB/p16INK4a, leads to uncontroled proliferation and finally to an increased clinical aggressiveness. Indeed, p16INK4a and p21CIP1/WAF1 act in the pathway of oncogene-induced CS, and the activation of this mechanism during tumor progression limits cancer progression (46). As CS was reported to be induced by various chemotherapeutic drugs (20, 21), it is important to note that in our study, the CS markers associated with a favorable outcome were present before treatment. These observations are particularly interesting because they present evidence with a prognostic value, wherein tumors containing the cells predisposed to CS could be more susceptible to CS induction by anticancer treatment. As such, these tumors could have a better prognosis.

Another possible explanation could be attributed to the recent observation that p16INK4a is a major barrier to proliferation in B cells transformed by EBV, a virus that has been implicated in the pathogenesis of cHL (47). The pathogenic role of EBV infection in cHL (48) is well known, and EBV has been found in HRS cells in about 40% of cases (10) and EBV may have a role in inhibiting p16INK4a expression (14). We also observed only a trend between the expression of p16INK4a and the presence of EBV (data not shown). Notably, the viral latent membrane protein 1 (LMP1, a marker of EBV presence) blocks p16INK4a expression (49).

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. of patients (%)</th>
<th>EFS % plateau</th>
<th>95% CI (%)</th>
<th>P*</th>
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<tbody>
<tr>
<td>Primary cohort</td>
<td>147</td>
<td>74</td>
<td>(55–86)</td>
<td>0.018</td>
</tr>
<tr>
<td>I-II</td>
<td>46 (31)</td>
<td>52</td>
<td>(23–75)</td>
<td></td>
</tr>
<tr>
<td>p16* /p21</td>
<td>14 (33)</td>
<td>76</td>
<td>(36–94)</td>
<td></td>
</tr>
<tr>
<td>p16* /p21</td>
<td>12 (26)</td>
<td>100</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Bulky</td>
<td>42 (29)</td>
<td>62</td>
<td>(44–75)</td>
<td></td>
</tr>
<tr>
<td>p16* /p21</td>
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</tr>
<tr>
<td>p16* /p21</td>
<td>14 (33)</td>
<td>76</td>
<td>(42–92)</td>
<td></td>
</tr>
<tr>
<td>p16* /p21</td>
<td>13 (31)</td>
<td>100</td>
<td>–</td>
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</tr>
<tr>
<td>III-IV</td>
<td>59 (40)</td>
<td>62</td>
<td>(48–74)</td>
<td></td>
</tr>
<tr>
<td>p16* /p21</td>
<td>18 (31)</td>
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<tr>
<td>p16* /p21</td>
<td>19 (32)</td>
<td>76</td>
<td>(47–90)</td>
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<tr>
<td>p16* /p21</td>
<td>22 (37)</td>
<td>88</td>
<td>(59–97)</td>
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<tr>
<td>Validation cohort</td>
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<tr>
<td>I-II</td>
<td>42 (46)</td>
<td>85</td>
<td>(63–99)</td>
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</tr>
<tr>
<td>p16* /p21</td>
<td>9 (21)</td>
<td>50</td>
<td>(14–79)</td>
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<tr>
<td>p16* /p21</td>
<td>11 (26)</td>
<td>82</td>
<td>(45–95)</td>
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<td>22 (52)</td>
<td>100</td>
<td>–</td>
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<td>Bulky</td>
<td>16 (18)</td>
<td>66</td>
<td>(35–85)</td>
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<tr>
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<td>100</td>
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<tr>
<td>p16* /p21</td>
<td>14 (42)</td>
<td>100</td>
<td>–</td>
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</table>

NOTE: Statistically significant P values are shown in bold type; –, < 30% or –, ≥50% HRS cells expressing the indicated molecule; *, either molecule <30%.

*For comparison between p16INK4a/p21CIP1/WAF1 groups.
In this study, we demonstrate the potential value of immuno-histochemical detection of p16\(^{ink4a}\) and p21\(^{cip1/waf1}\) in diagnostic samples for the prediction of CHL outcome. In our series, the cases of CHL exhibiting absence or low expression of the CS markers p21\(^{cip1/waf1}\) and p16\(^{ink4a}\) in HRS cells tended to relapse or did not respond to therapy. Given the simple and reproducible immunohistochemical approach described in this article, we would like to propose a rapid and low-cost method, which could be applied in routine diagnosis. Although further and prospective studies are warranted to confirm our data, the results of our study support the hypothesis that CS features may be characteristic of HRS cells in the majority of CHL.

Disclosure of Potential Conflicts of Interest

A. Zamö reports receiving speakers bureau honoraria from Novartis. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Caliö, A. Zamö, M. Ponsoni, A.J.M. Ferrari, C. Doglioni
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Caliö, A. Zamö, M.E. Zanolin, V.E. Fraifeld, M. Wolfson, H. Yanai, C. Doglioni, F. Vinante
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Anna Caliò, Alberto Zamò, Maurilio Ponzoni, et al.


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