

# Aberrant expression of cell cycle regulators in Hodgkin and Reed–Sternberg cells of classical Hodgkin's lymphoma

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The characteristic Hodgkin and Reed–Sternberg cells of classical Hodgkin's lymphoma, although highly positive for proliferation markers, do not accumulate to excessive cell numbers. These cells are characterized by abortive mitotic cycles, leading to multinucleation or cell death in mitosis. We have previously described high expression of G<sub>1</sub>-phase cyclins in classical Hodgkin's lymphoma, which could explain the high percentage of cells staining for proliferation markers. To further our understanding of proliferation control in classical Hodgkin's lymphoma, we extended our immunohistochemical analysis to the main S-phase cyclin, cyclin A, and its regulators p21<sup>CIP1</sup> and p27<sup>KIP1</sup>. Expression of proliferating cell nuclear antigen (PCNA) was used as an additional marker for cells being in either S- or G<sub>2</sub>-phase. In 47% (112/239) of classical Hodgkin's lymphoma cases p21<sup>CIP1</sup> was detected within a mean frequency of 15% positive Hodgkin's and Reed–Sternberg cells per case. Similarly, 47% (116/249) of the cases stained positively for p27<sup>KIP1</sup> with a mean frequency of expression in Hodgkin's and Reed–Sternberg cells of 12%. In contrast, 90% of the cells in all 246 evaluable classical Hodgkin's lymphoma cases were positive for PCNA. In addition, 98% of Hodgkin's and Reed–Sternberg cells in 99% (250/253) of the cases stained strongly positive for cyclin A. These findings further corroborate the hypothesis that Hodgkin and Reed–Sternberg cells exhibit a disturbed cell cycle with an abnormally short or even absent G<sub>1</sub>-phase. In contrast to other tumors, expression of PCNA or cyclin A had no prognostic value for patient survival.

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The Hodgkin and Reed–Sternberg cells of classical Hodgkin's lymphoma exhibit a proliferation defect caused by abortive mitotic cycles, the latter characterized by abnormal metaphase to anaphase transitions as well as by cytokinesis defects, which may cause multinucleation, polyploidy or mitotic cell death.<sup>1,2</sup> This mitotic defect could at least partially explain the discrepancy between the high expression of proliferation-associated antigens, such as Ki-67, in Hodgkin and Reed–Sternberg cells and the concomitant lack of successful tumor-cell

production.<sup>2–4</sup> In addition, Reed–Sternberg cells are polyploid and probably amplify their genome by skipping cytokinesis to repeatedly switch between S-phase and mitosis.<sup>5</sup>

We recently demonstrated alterations in the expression of G<sub>1</sub>-phase cyclins in classical Hodgkin's lymphoma, particularly that of cyclin E,<sup>6</sup> the high expression of which led us to conclude that Hodgkin and Reed–Sternberg cells either have a particularly long G<sub>1</sub>-phase or express an abnormally stable cyclin E, which is not degraded in S-phase cells. To distinguish between these possibilities, we investigated genes known to be expressed in non-proliferating cells, such as the cyclin-dependent kinase inhibitors p27<sup>KIP1</sup> and p21<sup>CIP1</sup>, as well as genes typically expressed in proliferating cells, such as cyclin A and proliferating cell nuclear antigen (PCNA).

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The cyclin-dependent kinase inhibitors p27<sup>KIP1</sup> and p21<sup>CIP1</sup> act by binding to and inhibiting the cyclin E and cyclin A-activated cyclin-dependent kinase 2, thus controlling entry into S-phase.<sup>7-9</sup> Both cyclin-dependent kinase inhibitors are controlled at multiple levels and coordinate cellular proliferation by integrating extracellular as well as intracellular cues (reviewed in Sherr and Roberts<sup>10</sup>). In contrast to p27<sup>KIP1</sup>, which becomes unstable once cells enter S-phase, p21<sup>CIP1</sup> can be induced at any time during the cell division cycle in p53-dependent as well as independent manners, and controls not only the G<sub>1</sub>-to S-phase transition, but also progression through S-phase as well as entry into mitosis.<sup>7,9,11,12</sup> During S-phase, p21<sup>CIP1</sup> is able to associate with the DNA clamp protein PCNA to control the activity of DNA polymerases.<sup>8,10</sup>

Progression through S- and G<sub>2</sub>-phase and entry into mitosis are controlled by cyclin A, which becomes detectable as cells enter S-phase (reviewed in Gillett and Barnes<sup>13</sup> and Sherr<sup>14</sup>). Cyclin A levels and associated cyclin-dependent kinase 2 activity steadily increase during S-phase until prometaphase, when cyclin A becomes an unstable protein.<sup>15,16</sup> Cyclin A can, therefore, be regarded as a marker for cells in S- or G<sub>2</sub>-phase of the cell cycle. Similarly, PCNA is extensively used (together with Ki-67) as a marker for cells in S- or G<sub>2</sub>-phase of the cell division cycle.

To investigate the mechanisms that control cell cycle progression during the S- and G<sub>2</sub>-phase and to further characterize cell cycle regulation in Hodgkin and Reed–Sternberg cells, we performed immunohistochemical analyses of p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, cyclin A and PCNA expression on our previously validated tissue microarray encompassing 330 cases of classical Hodgkin's lymphoma.<sup>6,17</sup>

## Materials and methods

### Patients

A total of 330 paraffin-embedded classical Hodgkin's lymphoma tissue samples<sup>6</sup> were included in this study, covering all histological subtypes of the World Health Organization classification.<sup>18</sup> They comprised 197 nodular sclerosis, 105 mixed cellularity, 10 diffuse and nodular lymphocyte-rich and five lymphocyte-depleted as well as 13 unclassifiable classical Hodgkin's lymphoma cases. In 107 patients, aged between 12 and 87 years, clinical follow-up data were obtained by reviewing the charts (Table 1). Treatment was either standard or consistent with the investigational protocols active during the time the patients were diagnosed. Disease remission was defined as absence of disease for at least 1 month after the last treatment regimen ended, as assessed by laboratory and imaging studies and physical examination. Disease relapse was defined as disease progression after at least 1 month of disease remission. Nine patients died due to treat-

**Table 1** Clinicopathological characteristics of classical Hodgkin's lymphoma patients with follow-up

Patients (n)	n	%
<i>Histology (107)</i>		
Nodular sclerosis	63	59
Mixed cellularity	32	30
Lymphocyte-depleted	3	3
Lymphocyte-rich	1	1
Unclassified	8	7
<i>Sex (107)</i>		
Male	56	52
Female	51	48
<i>Ann-Arbor stage (91)</i>		
I–II	55	61
III–IV	36	39
Mean age (years) (107)	35.5 (12–87)	
<i>Therapy (102)</i>		
Radiotherapy	23	23
Chemotherapy/radiochemotherapy	79	79
B-symptoms (99)	39	40
Median follow-up, months (107)	145 (5–331)	
Dead with/on disease (107)	9	8
Disease relapses (102)	28	26

ment failures (eight relapses, and one resistant disease), 11 due to histologically documented second malignancies without evidence of persistent Hodgkin's lymphoma and seven due to cardiovascular emergencies. Within the median follow-up period of 145 months, cumulative disease-specific survival was 92%, whereas overall survival was 75%.

### Construction of Tissue Microarrays and Morphological Analysis

The construction of tissue microarray was performed as described previously.<sup>6,17</sup> Sections of the tissue microarray blocks, 4 μm thick, were transferred to an adhesive-coated glass slide system (Instrumedics Inc., Hackensack, NJ, USA) and stained with hematoxylin and eosin, Giemsa and with the periodic acid Schiff reaction. Only cases containing at least two morphologically unequivocal Hodgkin and Reed–Sternberg cells were analyzed.

### Immunohistochemistry

Bound secondary antibodies were visualized by standard avidin–biotin–peroxidase technique using diaminobenzidine as chromogene. Commercially available primary antibodies against p21<sup>CIP1</sup> (DAKO, dilution 1:40), p27<sup>KIP1</sup> (DAKO, dilution 1:100), PCNA (DAKO, dilution 1:300) and cyclin A (Neomarkers, dilution 1:500) were applied. Nuclear staining

was quantified as percentage of positive Hodgkin and Reed–Sternberg cells' nuclei in out of all detectable tumor cells. To estimate the prognostic value of cyclin-dependent kinase inhibitors, the cases were grouped in negative and positive cases with a cutoff level of expression in  $\leq 10\%$  of the Hodgkin and Reed–Sternberg cells, or, in cases with less than 10 Hodgkin and Reed–Sternberg cells,  $\leq 50\%$ . In the negative control experiments, the primary antibodies were omitted.

**Statistics**

Statistical analysis was performed applying the Statistical Package of Social Sciences. The Spearman's rank correlation and the Pearson's  $\chi^2$  tests were used to demonstrate correlations between the expression of p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, PCNA and G<sub>1</sub>-cyclins as well as association with Epstein–Barr virus-infection. Disease-specific- and overall survival were analyzed by the Kaplan–Maier method and compared by the log-rank test. Multivariate analysis for the prognostic significance of the expression of p21<sup>CIP1</sup>, p27<sup>KIP1</sup> and PCNA as well as for age, sex, Ann-Arbor stage, Epstein–Barr virus-infection, B-symptoms, disease relapses and therapy was performed using a Cox stepwise regression model. P-values below 0.05 were considered significant.

**Results**

**Histopathology**

Of 330 classical Hodgkin's lymphoma cases included in the tissue microarrays, 260 (79%) were representative by hematoxylin and eosin morphology. The analysis failure of 70 cases (21%) was linked to problems associated with the array technology, as discussed recently.<sup>6,17</sup> The evaluable cases consisted of 152 nodular sclerosis, 84 mixed cellularity, nine lymphocyte-rich, five lymphocyte-depleted and 10 unclassifiable classical Hodgkin's lymphomas comprising a total mean number of 10243 Hodgkin and Reed–Sternberg cells per slide. Five cases of nodular sclerosis classical Hodgkin's lymphoma were inadequately fixed and, therefore, immunohistochemical examinations could be performed on 147 nodular sclerosis cases. Cores containing 'no tissue' varied from slide to slide. Thus, the evaluation of cases containing diagnostic Hodgkin and Reed–Sternberg cells changed accordingly compared to previous studies with these tissue microarrays.<sup>6,17</sup>

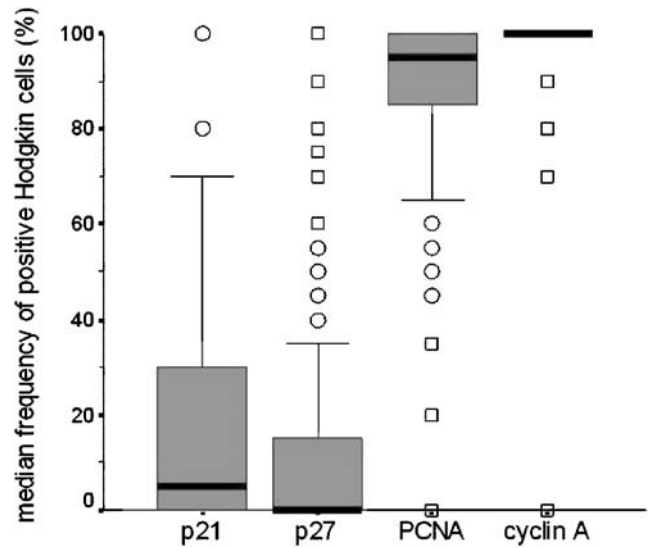
**Immunohistochemical Analysis**

In 47% (112/239) of classical Hodgkin's lymphoma samples, the mean frequency of p21<sup>CIP1</sup> expression in Hodgkin and Reed–Sternberg cells per positive case was 15% (95% confidence interval 14–19%,

standard deviation  $\pm 22\%$ ). Some of the tumor samples stained highly positive for p21<sup>CIP1</sup>, giving rise to a wide variability of p21<sup>CIP1</sup> expression in Hodgkin and Reed–Sternberg cells (Figure 1, Table 2). The staining intensity was moderate to strong and diffusely distributed throughout the nucleus (Figure 2a). Among the reactive lymphocytes surrounding Hodgkin and Reed–Sternberg cells, very few expressed p21<sup>CIP1</sup>.

Of the classical Hodgkin's lymphoma cases, 47% (116/249) expressed p27<sup>KIP1</sup> in 12% (mean frequency) of Hodgkin and Reed–Sternberg cells (95% confidence interval 10–16%, standard deviation  $\pm 23\%$ ) (Figure 1, Table 2). The staining intensity was weak to moderate and diffusely distributed throughout the nucleus (Figure 2b). The background reactive small lymphocytes expressed p27<sup>KIP1</sup> with a moderate to strong staining intensity in over 80% of the cell nuclei.

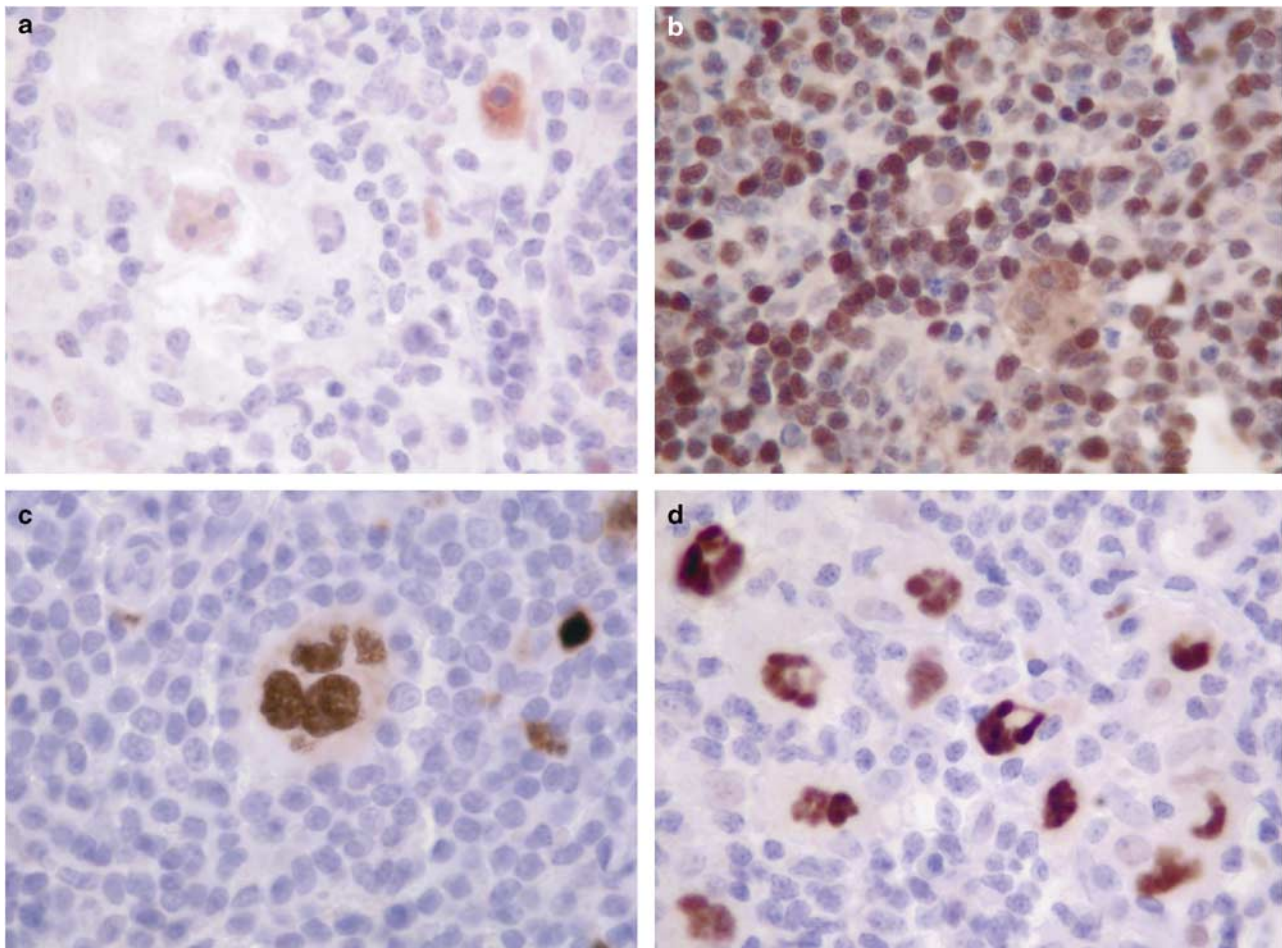
Of the classical Hodgkin's lymphomas, 99% (250/253) expressed cyclin A in the vast majority of Hodgkin and Reed–Sternberg cells (mean frequency 98%, 95% confidence interval 97–99%, standard deviation  $\pm 14\%$ ) (Figure 1, Table 2). Only in three tumor samples, Hodgkin and Reed–Sternberg cells were found to be negative for cyclin A. The staining intensity was strong and diffusely distributed throughout the nucleus (Figure 2c). Some surrounding lymphocytes stained positively with the anti-cyclin A antibody.



**Figure 1** Median expression of the cell cycle regulating proteins p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, PCNA and cyclin A in Hodgkin and Reed–Sternberg cells of classical Hodgkin's lymphoma, box-plot, the box length being the interquartile range, cases with values 1.5–3 (outliers ○) or > 3 (extremes □) box lengths from the upper or lower edge of the box are designated separately. p21<sup>CIP1</sup> median expression 5%, mean 15%, upper quartile 25%, lower quartile 0%, range 0–100%; p27<sup>KIP1</sup> median expression 0%, mean 12%, upper quartile 15%, lower quartile 0%, range 0–100%; PCNA median expression 95%, mean 90%, upper quartile 100%, lower quartile 85%, range 0–100%; cyclin A median expression 100%, mean expression 98%, upper quartile 100%, lower quartile 100%.

**Table 2** p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, PCNA and cyclin A expression in classical Hodgkin's lymphoma: mean proportion of positive Hodgkin's and Reed–Sternberg cells

Classical Hodgkin's lymphoma subtype (n)	Mean proportion of positive Hodgkin and Reed–Sternberg cells (%) ( $n^{positive}/n^{evaluable}$ cases)			
	p21 <sup>CIP1</sup>	p27 <sup>KIP1</sup>	PCNA	Cyclin A
Nodular sclerosis (147)	15 (55/136)	11 (57/142)	90 (141/141)	98 (143/146)
Mixed cellularity (84)	19 (47/81)	17 (51/83)	91 (83/83)	97 (83/83)
Lymphocyte-rich (9)	13 (2/8)	11 (3/9)	66 (8/8)	100 (9/9)
Lymphocyte-depleted (5)	14 (2/5)	26 (4/5)	95 (5/5)	100 (5/5)
Unclassifiable (10)	14 (6/9)	10 (4/10)	82 (9/9)	100 (10/10)
Total (255)	15 (112/239)	12 (116/249)	90 (246/246)	98 (250/253)



**Figure 2** (a) Expression of p21<sup>CIP1</sup> in a Hodgkin cell. Note the negative surrounding lymphocytes as well as a negative Hodgkin cell (original magnification  $\times 200$ ). (b) Expression of p27<sup>KIP1</sup> in a Reed–Sternberg cell. Note the strong staining intensity in surrounding lymphocyte nuclei (original magnification  $\times 200$ ). (c) Expression of cyclin A in a Reed–Sternberg cell (original magnification  $\times 250$ ). (d) Expression of proliferating cell nuclear antigen in Hodgkin and Reed–Sternberg nuclei (original magnification  $\times 200$ ).

All 246 evaluable classical Hodgkin's lymphomas expressed PCNA in a mean proportion of 90% of Hodgkin and Reed–Sternberg cells (95% confidence interval 87–91%, standard deviation  $\pm 16\%$ ) (Figure 1, Table 2). PCNA staining was more variable than

the staining for cyclin A. Only few surrounding lymphocytes were found to be positive for PCNA (Figure 2d).

The observed staining patterns in the reactive surrounding lymphocytes gave the expected staining

patterns and frequencies, indicating that the antibodies and staining conditions employed in this study were appropriate for detection of these proteins.

**Statistical Analysis**

Statistical analysis (Table 3) revealed significant correlations between the expression of p21<sup>CIP1</sup> and p27<sup>KIP1</sup> ( $P < 0.001$ ) with 34% (80/235) of classical Hodgkin's lymphoma cases expressing both cyclin-dependent kinase inhibitors. Since the analysis of the expression of cyclin-dependent kinase inhibitors was performed on consecutive 4 μm sections, making analysis of coexpression in single cells difficult, we statistically calculated that approximately 500 of the analyzed 10 000 Hodgkin and Reed–Sternberg cells per slide (5%) expressed both inhibitors. The expression of these proteins and their frequency suggests that these tumor cells are in G<sub>1</sub>-phase. PCNA expression, on the other hand, correlated with expression of cyclin A, with 99% (244/246) of classical Hodgkin's lymphoma cases being positive for both ( $P = 0.005$ ). Approximately 9000 of the average 10 000 Hodgkin and Reed–Sternberg cells present on every tissue microarray slide expressed cyclin A and PCNA, suggesting that more than 85% of these cells were in either S- or G<sub>2</sub>-phase. As expected from our previous analysis,<sup>6</sup> expression of PCNA also correlated with expression of cyclin D3 ( $P = 0.002$ ) and cyclin E ( $P < 0.0001$ ), suggesting that a significant fraction of S-phase Hodgkin and Reed–Sternberg cells are positive for cyclin E.

Univariate analysis revealed a significantly higher frequency of treatment failures in patients with stage III and IV disease (16/36, 44%) compared to those with stages I and II (12/64, 19%,  $P = 0.006$ ). The presence of B-symptoms correlated with clinical stage ( $P = 0.017$ ). Patient age correlated with the different causes of death, that is, those younger than 45 years were more likely to succumb to lymphoma, whereas patients older than 45 years died of secondary malignancies or cardiovascular events ( $P < 0.0001$ ).

Expression of the analyzed cell cycle regulatory proteins did not reach statistical significance for prognosis in classical Hodgkin's lymphoma, although only three of 54 patients expressing p21<sup>CIP1</sup> (6%) died of lymphoma, compared to six of 38 p21<sup>CIP1</sup>-negative patients (16%,  $P = 0.075$ ). Disease stage ( $P = 0.004$ ) was of independent prognostic significance concerning failure-free survival in the multivariate analysis.

**Discussion**

Unlike most other malignant tumors, classical Hodgkin's lymphoma is not characterized by high numbers of proliferating tumor cells. In contrast, Hodgkin and Reed–Sternberg cells are sparse and surrounded by many activated non-neoplastic lymphocytes. Reed–Sternberg cells are multinucleated, while Hodgkin cells are often near triploid (reviewed in Chan<sup>19</sup>), which grow slowly *in vitro* (personal observations on HDLM-2 cell cultures). The slow growth phenotype of Hodgkin and Reed–Sternberg cells is accompanied by frequent mitotic defects as well as a high incidence of apoptosis.<sup>20</sup> This suggests that Hodgkin and Reed–Sternberg cells might be actively proliferating cells that fail to accumulate due to an intrinsic failure to divide properly. Several proteins that regulate the mitotic cycle, such as cyclin D1, D3, E, B1, cyclin-dependent kinases 1, 2 and 6, S-phase kinase associated protein-2, p16<sup>INK4A</sup>, p18<sup>INK4C</sup>, p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, p53, the retinoblastoma protein and PCNA have been found to be deregulated in classical Hodgkin's lymphoma.<sup>1–4,6,20–26</sup>

In the present study, we used a previously validated classical Hodgkin's lymphoma tissue microarray with a cohort of clinically well-documented cases<sup>6,17,27</sup> to analyze the expression of the cyclin-dependent kinase inhibitors of the Cip/Kip-family, p21<sup>CIP1</sup> and p27<sup>KIP1</sup>, as well as two proliferation markers, PCNA and cyclin A. Since Hodgkin and Reed–Sternberg cells are embedded within reactive normal lymphocytes, we used those lymphocytes as internal controls for our staining

**Table 3** Correlations between expression of p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, PCNA, G<sub>1</sub>-cyclins and the latent membrane protein 1 of Epstein–Barr virus (LMP1) in classical Hodgkin's lymphoma (*P*-values (upper) and correlation coefficients (lower))

p27 <sup>KIP1</sup>	<0.001						
	0.208						
PCNA	—	—					
LMP1	—	—	—				
Cyclin A	—	—	0.005	—			
			0.181				
Cyclin D1	0.034	—	—	—	—		
	0.141						
Cyclin D3	—	—	0.002	0.006	0.018	0.003	
			0.194	0.173	0.150	0.187	
Cyclin E	<0.0001	—	<0.0001	—	—	0.016	<0.0001
	0.264		0.271			0.151	0.224
	p21 <sup>CIP1</sup>	p27 <sup>KIP1</sup>	PCNA	LMP1	cyclin A	cyclin D1	cyclin D3

conditions. As expected, most of these lymphocytes stained strongly for p27<sup>KIP1</sup>, while only a few stained for PCNA or cyclin A, suggesting that they are in the G<sub>1</sub>-phase of the cell cycle. p21<sup>CIP1</sup> was hardly detectable in these lymphocytes. In Hodgkin and Reed–Sternberg cells, however, we found significant divergences from the expected expression frequency of cyclin-dependent kinase inhibitors, PCNA and cyclin A.

In normal, as well as transformed, human cells, cyclin A becomes detectable at the onset of the S-phase, accumulates throughout S- and G<sub>2</sub>-phase, and then rapidly declines in early mitosis (reviewed in Gillett and Barnes,<sup>13</sup> den Elzen and Pines<sup>15</sup> and Geley *et al*<sup>16</sup>). Cyclin A can, therefore, be regarded as a marker of proliferation, since only cells in S- and G<sub>2</sub>-phase or early mitosis stain positively with anti-cyclin A antibodies, while cells in G<sub>1</sub>-phase remain negative. We found cyclin A expression in 98% of the Hodgkin and Reed–Sternberg cells in nearly all (99%) analyzed classical Hodgkin's lymphoma cases, suggesting that most of the tumor cells are in either S- or G<sub>2</sub>-phase of the cell cycle. This hypothesis was corroborated by PCNA staining experiments using our classical Hodgkin's lymphoma tissue microarray, which showed that 90% of Hodgkin and Reed–Sternberg cells were positive. Because PCNA is mainly expressed in S-phase cells, we conclude that the majority of Hodgkin and Reed–Sternberg cells are either in S- or G<sub>2</sub>-phase of the cell division cycle. Our data confirm previously published data<sup>2–4,22,24</sup> reporting high expression of PCNA and Ki-67 in classical Hodgkin's lymphoma.

The cyclin-dependent kinase inhibitors p27<sup>KIP1</sup> and p21<sup>CIP1</sup> were rarely expressed in Hodgkin and Reed–Sternberg cells, and such expression correlated well with each other as well as with the expression of G<sub>1</sub>-cyclins, suggesting that these few cells were in G<sub>1</sub>-phase. In contrast to our data, Garcia *et al*<sup>22</sup> and Ohshima *et al*<sup>25</sup> reported higher expression of p27<sup>KIP1</sup> and p21<sup>CIP1</sup> in their classical Hodgkin's lymphoma samples. However, Garcia *et al* applied other primary anti-p27<sup>KIP1</sup> antibodies, and Ohshima *et al* determined the expression of p21<sup>CIP1</sup> on frozen classical Hodgkin's lymphoma tissue samples, which might explain some of the differences. Our observed low expression levels of p21<sup>CIP1</sup> and p27<sup>KIP1</sup> is in agreement with reports by other authors.<sup>3,23,24</sup>

Our data suggest that about 80–90% of Hodgkin and Reed–Sternberg cells are in S- or G<sub>2</sub>-phase, while only about 10–20% are in G<sub>1</sub>-phase. Surprisingly, however, we could only count 57 mitoses in 10 243 cells analyzed. This low mitotic index (0.5%) suggests that Hodgkin and Reed–Sternberg cells either do not enter mitosis or rapidly die in mitosis. Concerning the overexpressed G<sub>1</sub>-cyclins, including cyclin E, in Hodgkin's lymphoma, our present data suggest that their accumulation may be caused by reduced proteolysis, rather than being a reflection of typical cell cycle-dependent expression levels.

Hodgkin and Reed–Sternberg cells have large nuclei indicative of higher DNA content and, in our study, 20% exhibited a multinuclear Reed–Sternberg phenotype. In contrast to micronuclei, which are not observed in Hodgkin and Reed–Sternberg cells, and which are caused by chromosome malsegregation during mitosis, multinucleation is caused by cytokinesis defects or cell fusion. Koppers *et al*<sup>28</sup> have investigated the mechanism leading to multinucleation in Hodgkin and Reed–Sternberg cells and reported that it is not caused by cellular fusion. The large nuclei present in Hodgkin and Reed–Sternberg cells may, therefore, be caused by cytokinesis defects or by DNA over-replication. Whether the former or the latter underlies the aberrant morphology and biology of Hodgkin and Reed–Sternberg cells is an important unresolved issue. Expression of cyclin E is required for endoreduplication in trophoblasts<sup>29</sup> as well as in megakaryocytes<sup>30</sup> and overexpression of cyclin E has been correlated with polyploidy and chromosome instability in human tumors (Hubalek *et al*<sup>31</sup> and references therein). Whether and how overexpression of cyclin A might contribute to the cell cycle abnormalities observed in Hodgkin and Reed–Sternberg cells is not clear at the moment. Overexpression of cyclin A has been shown to impede progression through mitosis and might facilitate apoptosis.<sup>32</sup> The expression of cell cycle regulatory proteins in malignant tumors is often analyzed to obtain prognostic information. High expression of proliferation markers, including Ki-67, PCNA, cyclin A and others, often have been associated with poor prognosis due to the high proliferation rate of the tumors. In classical Hodgkin's lymphoma, however, we found no association between cell cycle markers and clinical outcome. Again, this reflects the atypical nature of this disease, the pathology of which is not caused by rapid accumulation of malignant cells, but rather by the accumulation of reactive lymphocytes and perturbation of the cellular and humoral immunity (reviewed in Skinnider and Mak<sup>33</sup>). We found a weak correlation between p21<sup>CIP1</sup> expression and disease-specific survival, which may be caused by induction of p53-dependent apoptotic pathways in a subset of classical Hodgkin's lymphomas.<sup>21,34</sup>

In summary, our study has shown that the majority of reactive lymphocytes surrounding the tumor cells of classical Hodgkin's lymphoma are in G<sub>1</sub>-phase, while the majority of Hodgkin and Reed–Sternberg cells appear to be either in S- or G<sub>2</sub>-phase of the cell cycle. These data argue that self-replication of Hodgkin and Reed–Sternberg cells is futile with cells unable to divide properly. Rather, these cells acquire polyploidy due to possible defects in cytokinesis or activation of endomitotic cycles causing over-replication of DNA. Further *in vitro* studies using classical Hodgkin's lymphoma derived cell lines will distinguish between these possibilities.

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