

## Original Article

## High Expression of Minichromosome Maintenance Protein 6 in Classic Hodgkin's Lymphoma Points to a Cell Cycle Arrest in G1 Phase

Shirin Karimi MD\*, Forouzan Mohammadi MD\*\*, Kian Khodadad MD\*\*\*,  
Habib Emami MSc\*, Leila Seyfollahi MD\*

**Background:** Minichromosome maintenance protein 6 (MCM6) is one of the six proteins of minichromosome maintenance family that are involved in the initiation of DNA replication and thus represent a marker for proliferating cells. The aim of this study was to determine the proliferation characteristics of neoplastic cells in patients with classic Hodgkin's lymphoma.

**Methods:** Paraffin-embedded blocks of lymph node, mediastinal, subcutaneous chest wall, and lung mass biopsies of 55 patients with classic Hodgkin's lymphoma were immunostained by the proliferation-associated monoclonal antibodies; Ki-S5 (Ki-67 antigen) and Ki-MCM6 (MCM6 antigen).

**Results:** High MCM6 antigen expression was a striking feature of Hodgkin's and Reed-Sternberg cells (median: 85%, range: 35 – 99%) in comparison with lower Ki-67 expression (median: 63.5%, range: 1 – 98%,  $P < 0.001$ ). This indicates that MCM6 is already expressed in the early G1 phase, a cell cycle fraction that is not covered by antibodies specific to the Ki-67 antigen. The proliferation rates were determined by two markers, independent of histologic subtype, stage, presence of B symptoms, and size.

**Conclusion:** These data show that a subset of Reed-Sternberg and Hodgkin's cells is arrested in the early G1 phase and the MCM6-positive cells do not necessarily represent the real proliferating compartment of Hodgkin's lymphoma. Clinical relevance of this marker in patients with Hodgkin's lymphoma should be investigated.

*Archives of Iranian Medicine, Volume 11, Number 5, 2008: 532 – 538.*

**Keywords:** Cell cycle • cell proliferation • Hodgkin's lymphoma • Ki-67 • MCM6

### Introduction

The role of proliferation rate of neoplastic cells in non-Hodgkin's lymphoma in classification and prediction of clinical course, survival, and response to chemotherapy has been well-documented. However, due to the lack of data from prospective trials and limited predictive power

of the markers available so far (e.g., Ki-67), cell proliferation has rarely been used for clinical decision making in Hodgkin's lymphoma.

Minichromosome maintenance (MCM) proteins play an important role in the replication of eukaryotic DNA by binding to chromatin before the initiation of DNA replication.<sup>1,2</sup> MCM6 is one of the six members of the MCM family,<sup>3</sup> and consists of 821 amino acids with a molecular mass of 105 kDa.<sup>4</sup> A specific monoclonal antibody has been developed against MCM6 (Ki-MCM6) that enables the accurate detection of MCM6 in paraffin-embedded tissue.<sup>4,5</sup> Using Ki-MCM6, it was shown that MCM6 is detectable in nucleolus or bound to nuclear chromatin during the entire cell cycle G1,

**Authors' affiliations:** \*Department of Pathology, \*\*Department of Oncology, \*\*\*Department of Epidemiology, National Research Institute of Tuberculosis and Lung Disease (NRITLD), Shaheed Beheshti University of Medical Sciences, Tehran, Iran.

•**Corresponding author and reprints:** Shirin Karimi MD, National Research Institute of Tuberculosis and Lung Disease (NRITLD), Poor-Ebtehaj Ave., Darabad, Tehran, Iran.

Tel: + 98-212-010-9490, Fax: + 98 212-010- 9680,

E-mail: shkarimi@nritld.ac.ir

Accepted for publication : 29 January 2008

S, G2, and M phases, but it is absent in G0 phase.<sup>4,6</sup> Despite this similar expression pattern of MCM6 and Ki-67 during the cell cycle phases (positive in G1, S, G2, and M phases), detailed cell cycle analysis reveals differences between both markers. During the early G1 phase, Ki-67 is undetectable, whereas MCM6 is expressed in the entire G1 phase. Therefore, a small subset of about 20% of proliferating cells in early G1 phase could be detected by MCM6 and not by Ki-67 in stimulated peripheral blood mononuclear cells.<sup>4</sup>

The clinical relevance of MCM proteins as proliferation markers has been investigated by immunohistochemistry in several different malignant tumors.<sup>7</sup> For example, in nonsmall cell lung cancer,<sup>8</sup> prostate cancer,<sup>9,10</sup> oral squamous cell carcinoma,<sup>11</sup> chondrosarcoma,<sup>5</sup> oligodendroglial tumors,<sup>12,13</sup> esophageal neoplasm,<sup>14</sup> renal cell carcinoma,<sup>15</sup> breast cancer,<sup>16</sup> endometrial carcinoma,<sup>17</sup> and thyroid carcinoma.<sup>18</sup> Most of these studies focused on the detection of MCM2.<sup>7,11,19-21</sup> So far only few investigations studied MCM6 expression.<sup>5,6</sup>

The aim of this study was to investigate MCM as a new proliferation marker in patients with Hodgkin's lymphoma.

We investigated proliferation index (PI) immunohistochemically by MCM6 and compared it with PI by Ki-67 and assessed their correlation with clinical parameters including stage, subtypes, age, sex, symptoms, site, and the size of the mass.

## Materials and Methods

Paraffin-embedded blocks of lymph node, mediastinal, subcutaneous chest wall, and lung mass biopsies of patients with Hodgkin's lymphoma that referred to our pathology department, were studied. There were 56 paraffin-embedded samples from 55 patients; one patient had recurrence.

### Study design

We performed an analytical cross-sectional study in which the proliferation indices of two proliferative markers, MCM6 and Ki-67, were compared with each other and their correlation with stage and subtypes of Hodgkin's lymphoma was assessed in 55 patients.

### Patients and samples

Biopsies from 55 patients (22 men and 33 women) with proved classic Hodgkin's lymphoma

(CHL) who had a mean age of 26 years (SD=11, range: 13 – 68) were investigated. Clinical data including sex, age, site, stage, B symptoms, and the size of the mass were available on oncology department of our center. The clinicopathologic characteristics of the patients are shown in Table 1.

### Histology and immunohistochemistry

All of the biopsy specimens were reviewed in the pathology department of our center. The diagnosis was made based on both hematoxylin and eosin (H&E)-stained slides and previous immunohistochemistry staining for CD30, CD15, LCA, CD20, and CD3 from the archive. They were classified according to the Rye classification,<sup>22</sup> and modified according to the WHO criteria.<sup>23</sup>

The specimens were investigated by immunohistochemistry with monoclonal antibodies against MCM6 and Ki-67. Monoclonal antibody against MCM6 had been prepared from Department of Hematopathology and lymph node registry, Kiel, Germany (with the permission of Professor R. Parwaresch), and Ki-S5 was prepared from DAKO (DakoCytomation Company, Denmark). For immunohistochemistry 4 – 5  $\mu$ m thick sections of paraffin-embedded, formalin-fixed tissue were mounted on 3-amino-propyl-

**Table 1.** Clinicopathologic characteristics of 55 patients with Hodgkin's lymphoma.

Clinicopathologic characteristics	Number (%)	Total
Age		55
Range	13 - 68	
Mean	26	
SD	11	
Sex		55
Female	22 (40 %)	
Male	33 (60 %)	
Symptom		52
A (without B symptoms)	6 (11.5 %)	
B	46 (88.5)	
Site		55
Lymph node	39 (71 %)	
Mediastinum	12 (22 %)	
Lung	2 (3.6 %)	
Subcutaneous	2 (3.6 %)	
Stage		52
I	1 (2 %)	
II	21 (40 %)	
III	18 (34.6 %)	
IV	12 (23 %)	
Subtype		55
Lymphocyte rich	2 (3.6 %)	
Mixed cellularity	16 (29 %)	
Nodular sclerosis	37 (67 %)	

triethoxy-silane pretreated slides. After deparaffinization and peroxidase pretreatment blocking, antigen retrieval was achieved by boiling the sections in Tris buffer, pH=9 in autoclave (1.1 atmosphere, 121°C for 10 min). Then the slides were incubated for 60 min at room temperature with the primary antibodies: Ki-S5, directed against Ki-67 antigen (supernatant, dilution 1:30) and Ki-MCM6 directed against MCM6 protein (supernatant, dilution 1:25). Staining was completed with the LSAB2 kit (DAKO, DakoCytomation Company, Denmark) and visualized with diaminobenzidine.<sup>24</sup> The previously stained slides for CD30 of these blocks were reviewed for unequivocal identification of neoplastic cells.

To evaluate the proliferation rate, the number of Ki-S5 or Ki-MCM6-positive tumor cells in a minimum of 10 high-power fields was counted. In each stained section at least 50 cells were counted. The number of positively immunostained Hodgkin's and Reed-Sternberg cells was compared with the total number of Hodgkin's and Reed-Sternberg cells. The tumor cell distribution within the lymphoid tissue was heterogeneous; for example partial infiltration in CHL of mixed cellularity type and in all of the cases, the background lymphocytes and histiocytes were shown to be positive for both these antibodies. To more accurately determine the number of proliferating cells, the expression ratio of Ki-MCM6 to Ki-S5 was calculated based on immunopositivity for both the antibodies. The Hodgkin's and Reed-Sternberg cells with any degree of clear nuclear staining, were counted as positive and the percentage was calculated blindly. Tonsil tissue was used as positive controls. Negative control samples were incubated with serum instead of the primary antibody.

### Statistical analysis

For the statistical analysis, the Wilcoxon test was used to compare MCM6 and Ki-67 distribution. For categorical variables, the  $\chi^2$  test was used. Student's *t*-test, variance analysis, and Mann-Whitney test were used for comparison between the mean percentile of MCM6 and Ki-67. *P* value  $\leq 0.05$  was considered significant.

## Results

MCM6 and Ki-67 nuclear expression was detectable in Hodgkin's and Reed-Sternberg cells

in all 55 cases with median proliferation rate of 85% (range: 35 – 99%) and 63.5% (range: 1 – 98%), respectively. A major finding of this study was that the expression of MCM6 protein was significantly higher than that of Ki-67 antigen, with median growth fraction of 21.5% (Figure 1). As is seen in all analyses performed, the Ki-67 expression shows more dispersion than MCM6 expression.

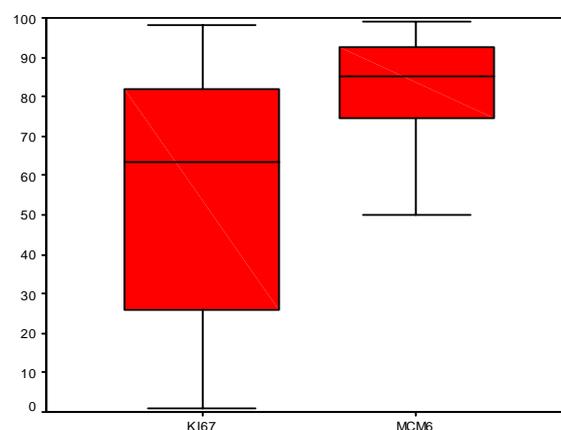
The median proliferation rate (MCM6 antigen) in nodular sclerosis was 81% (range: 40 – 99%). This rate was 89% (range: 35 – 96%) in the mixed cellularity subtype. This difference was not statistically significant.

No preference towards a histologic subtype was observed (Table 2).

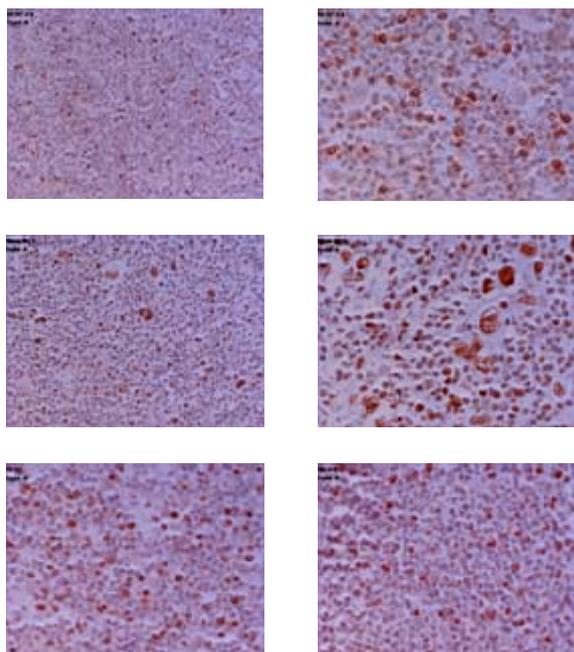
The staining intensity was generally stronger for Ki-MCM6, but positive and negative Hodgkin's, Reed-Sternberg, lymphocytic, and histiocytic cells could be differentiated in all cases (Figure 2). Another important finding was that the background small lymphocytes and histiocytes showed stronger Ki-67 positivity than MCM6 (Figure 3). The positive staining for Ki-MCM6 was also more homogeneous in neoplastic population (Figure 4). Similar to the staining results with the Ki-S5 antibody, no differences were detectable between different histologic subtypes.

The clinical data of 55 patients were evaluated. With regard to the growth fraction, no differences were observed between male and female patients. The proliferation rates in this study did not differ between the patients with localized (stage I/II) and advanced (stage III/IV) forms of the disease (Table 3).

Median percentages of Ki-MCM6-positive



**Figure 1.** Comparison between MCM6 and Ki-67 expression (median and range).

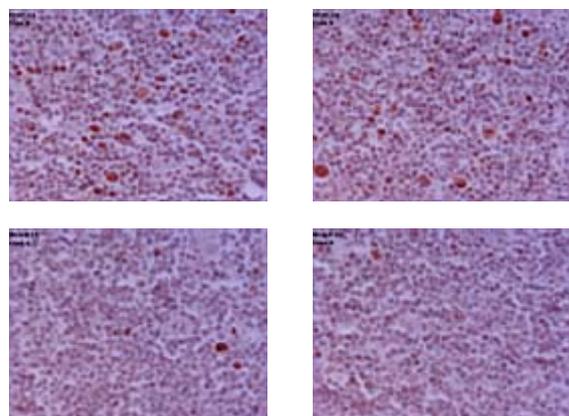


**Figure 2.** The nuclear positivity of Reed-Sternberg and mononuclear Hodgkin's cells for Ki-67 and Ki-MCM6 are evident. The intensity was prominently stronger for Ki-MCM6.

Hodgkin's and Reed-Sternberg cells, and Ki-67 expression according to the stage of disease, patients' symptoms, size, and site of the mass are shown in Table 3.

### Discussion

Tumoral cells in Hodgkin's lymphoma display an increased growth fraction and diminished apoptosis.<sup>25</sup> High Ki-67 antigen expression has been repeatedly described in Hodgkin's and Reed-Sternberg cells,<sup>26-31</sup> which are the putative neoplastic cells of this lymphoma, comprising less than 1% of all cells of the tumor.



**Figure 3.** The background small lymphocytes as well as neoplastic cells showed nuclear positivity for both Ki-67 and MCM6.

This finding, however, contrasts with the paucicellular nature and clinical behavior of this enigmatic lymphoma.<sup>25,32</sup>

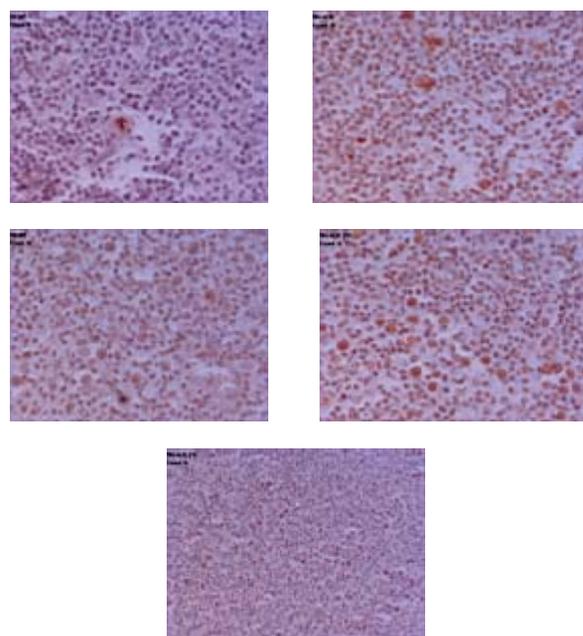
For the assessment of proliferation, the antigens under investigation must be restricted to proliferating cells or there must be a cell cycle-induced increase in their expression.

Immunohistochemistry has an advantage over flowcytometry in that cellular morphology and histology can be more accurately interpreted. The scarcity of Hodgkin's and Reed-Sternberg cells and the high proliferation rate of bystander cells make a reliable assessment of proliferation data difficult by means of flowcytometry technique.<sup>33</sup>

Since the development of monoclonal antibodies against formalin-resistant epitopes of Ki-67 antigen, the previously reported difficulties with poor morphology due to frozen tissues have been overcome. The most available antibody that is directed against proliferation-associated antigens (Ki-67) does not express in the early G1 phase of the cell cycle. In contrast, the monoclonal antibody Ki-MCM6 detects nuclear protein (MCM6) that is expressed in the G1, G2, S, and M phases, completely.

In our study, we also employed two previous studies, one focused on MCM7 in cervical cancer and the other focused on MCM6 in chondrosarcoma using the monoclonal antibody Ki-MCM6.<sup>5,34</sup>

We chose MCM6 for our analysis because



**Figure 4.** In comparison with Ki-67, the positive staining for Ki-MCM6 was more homogeneous in neoplastic population.

**Table 2.** MCM6 and Ki-67 expression and MCM6/Ki-67 ratio in relation to histologic subtypes in all 55 patients with Hodgkin's lymphoma.

Histology	N	MCM6 median (range)	Ki-67 median (range)	MCM6 / Ki-67 median (range)
LR	2	76 (56 – 96)	45 (1 – 89)	—
MC	16	89 (35 – 96)	62 (11 – 91)	1.2 (0.84 – 7.9)
NS	38	81 (40 – 99)	63.5 (1 – 98)	1.33 (0.8 – 82)

LR=lymphocytic rich; MC=mixed cellularity; NS=nodular sclerosing.

highly reliable monoclonal antibody against this member of MCM family was available.

Our study revealed that MCM6 expression was more than Ki-67 expression in neoplastic cells. For explaining this finding, we should consider other previous studies about the proliferation markers in CHL. The most important one is about Ki-S2.

The monoclonal antibody Ki-S2 detects a nuclear protein (repp86) that is expressed in the G2, S, and M phases, but not in the G1 phase. It enables the interpretation of individual cell cycle phase.<sup>35</sup> Tiemann et al. revealed that repp86 expression provided more accurate evidence of proliferating Hodgkin's and Reed-Sternberg cells than Ki-67 expression in serial sections of the same diagnostic lymph node (median Ki-67: 80%, median repp86: 20%,  $P<0.001$ ). They evaluated the PI both in neoplastic cells and background small lymphocytes and histiocytes, but we studied PI only in neoplastic cells.<sup>33</sup>

Comparison between these two above studies in neoplastic cells in CHL, could be possibly explained by G1 phase especially early G1 arrest which is of variable duration.<sup>33</sup>

These data are also in line with the results of the study on MCM6 and repp86 in a large series of patients with mantle cell lymphoma.<sup>36,37</sup> The MCM expression in peripheral B-cell lymphomas was investigated for the first time by Obermann et al.

who could demonstrate that also in mantle cell lymphomas (MCLs), the majority of lymphoma cells resided in the cell cycle phase G1, but not in S, G2, and M phases.<sup>38</sup>

Some authors believe that the discrepancy between high proliferation indices determined by proliferation markers and genuine growth factor of neoplastic cells in CHL, could be explained by occurrence of endomitoses, resulting in complex and variable karyotype abnormalities.<sup>39,40</sup> Sequential analyses of chromosomal aberrations reveal an increasing chromosomal instability of the genome, but no arithmetic doubling of the chromosomes.<sup>41,42</sup> Also, they believed that endomitosis does not play a central role in proliferation of Hodgkin's and Reed-Sternberg cells.<sup>41,42</sup> If the process of endomitosis is of minor influence only, more attention should be focused on early G1 arrest as a possible underlying pathogenetic mechanism.

In our study, high Ki-67 and MCM6 antigen expressions in Hodgkin's and Reed-Sternberg cells were related to neither advanced clinical stages nor the presence of B symptoms, may reflect that these markers could not indicate growth fraction in tumoral cells and emphasize the possible role of unregulated cytokine production in Hodgkin's lymphoma.

Our findings offer another strong evidence of

**Table 3.** MCM6 and Ki-67 expression and MCM6/Ki-67 ratio related to clinical stages, symptoms, and mass size in patients with Hodgkin's lymphoma.

	N	MCM6 median (range)	Ki-67 median (range)	MCM6/Ki-67 median (range)
Stage				
I/II	22	85.5 (1 – 94)	71.5 (40 – 96)	1.1 (0.8 – 80)
III/IV	30	82 (35 – 99)	61 (1 – 98)	1.3 (0.84 – 82)
Symptoms				
A	6	89 (63 – 94)	64.5 (6 – 92)	1.3 (1.02 – 10.5)
B	46	82 (35 – 99)	64 (1 – 98)	1.2 (0.8 – 82)
Size				
Nonbulky	24	86.5 (35 – 99)	79.5 (5 – 98)	1.2 (0.8 – 15.8)
Bulky	28	81 (38 – 96)	46 (1 – 91)	1.4 (0.84 – 82)
Site				
Lymph node	39	85 (38 – 98)	58 (1 – 98)	1.3 (0.84 – 82)
Mediastinum	12	81 (65 – 99)	77.5 (1 – 97)	1.1 (0.8 – 80)
Others	4	89 (35 – 92)	59 (29 – 79)	1.2 (1.1 – 1.7)
(Lung , subcutaneous)				

the importance of early G1 arrest in the pathogenesis of CHL and suggest that MCM6 is not a real proliferation marker in this type of lymphoma. These findings including high proliferative activity as well as G1 phase arrest could possibly explain the clinical behavior of Hodgkin's lymphoma resembling a low-grade lymphoma rather than a high-grade non-Hodgkin's lymphoma.

Further research has to combine different markers of proliferation (e.g., repp86) and cell cycle arrest (e.g., MCM6) with markers of apoptosis to get insight in to biology and explain the heterogeneous therapeutic response of this disease.

## References

- 1 Fujita M, Kiyono T, Hayashi Y, Ishibashi M. *In vivo* interaction of human MCM heterohexameric complexes with chromatin. Possible involvement of ATP. *J Biol Chem.* 1997; **272**: 10928 – 10935.
- 2 Ogawa Y, Takahashi T, Masukata H. Association of fission yeast Orp1 and MCM6 proteins with chromosomal replication origins. *Mol Cell Biol.* 1999; **19**: 7228 – 7236.
- 3 Linder K, Gregan J, Montgomery S, Kearsey SE. Essential role of MCM proteins in premeiotic DNA replication. *Mol Biol Cell.* 2002; **13**: 435 – 444.
- 4 Heidebrecht HJ, Buck F, Endl E, Kruse ML, Adam-Klages S, Andersen K, et al. Ki-Mcm6, a new monoclonal antibody specific to Mcm6: comparison of the distribution profile of Mcm6 and the Ki-67 antigen. *Lab Invest.* 2001; **81**: 1163 – 1165.
- 5 Helfenstein A, Frahm SO, Krams M, Drescher W, Parwaresch R, et al. Minichromosome maintenance protein 6 (MCM6) in low-grade chondrosarcoma: distinction from enchondroma and identification of progressive tumors. *Am J Clin Pathol.* 2004; **122**: 912 – 918.
- 6 Labib K, Kearsey SE, Diffley JF. MCM2-7 proteins are essential components of prereplicative complexes that accumulate cooperatively in the nucleus during G1 phase and are required to establish, but not maintain, the S phase checkpoint. *Mol Biol Cell.* 2001; **12**: 3658 – 3667.
- 7 Freeman A, Morris LS, Mills AD, Stoeber K, Laskey RA, Williams GH, et al. Minichromosome maintenance protein as biological markers of dysplasia and malignancy. *Clin Cancer Res.* 1999; **5**: 2121 – 2132.
- 8 Ramnath N, Hernandez FJ, Tan DF, Huberman JA, Natarajan N, Beck AF, et al. MCM2 is an independent predictor of survival in patients with non-small-cell lung cancer. *J Clin Oncol.* 2001; **19**: 4259 – 4266.
- 9 Meng MV, Grossfeld GD, Williams GH, Dilworth S, Stoeber K, Mulley TW, et al. Minichromosome maintenance protein 2 expression in prostate: characterization and association with outcome after therapy for cancer. *Clin Cancer Res.* 2001; **7**: 2712 – 2718.
- 10 Padmanabhan V, Callas P, Philip G, Trainer TD, Beatty BG. DNA replication regulation protein MCM7 as a marker of proliferation in prostate cancer. *J Clin Pathol.* 2004; **57**: 1057 – 1062.
- 11 Kodani I, Osaki M, Shomori K, Araki K, Goto E, Ryoke K, et al. Minichromosome maintenance protein 2 expression is correlated with mode of invasion and prognosis in oral squamous cell carcinoma. *J Oral Pathol Med.* 2003; **32**: 468 – 474.
- 12 Wharton SB, Chan KK, Anderson JR, Stoeber K, Williams GH. Replicative MCM2 protein as a novel proliferation marker in oligodendrogliomas and its relationship to Ki-67 labeling index, histological grade and prognosis. *Neuropathol Appl Neurobiol.* 2001; **27**: 305 – 313.
- 13 Wharton SB, Hibberd S, Eward KL, Crimmins D, Jellinek DA, Levy D, et al. DNA replication licensing and cell cycle kinetics of oligodendroglial tumors. *Br J Cancer.* 2004; **91**: 262 – 269.
- 14 Going JJ, Keith WN, Neilson L, Stoeber K, Stuart RC, Williams GH. Aberrant expression of minichromosome maintenance protein 2 and 5, and Ki-67 in dysplastic squamous oesophageal epithelium and Barret's mucosa. *Gut.* 2002; **50**: 373 – 377.
- 15 Dudderidge TJ, Stoeber K, Loddo M, Atkinson G, Griffiths DF, Williams GH. Mcm2, Geminin, and Ki-67 definite proliferative state and rare prognostic markers in renal cell carcinoma. *Clin Cancer Res.* 2005; **11**: 2510 – 2517.
- 16 Gonzalez MA, Tachibana KE, Chin SF, Callagy G, Madine MA, Vowler SL, et al. Geminin predicts adverse clinical outcome in breast cancer by reflecting cell-cycle progression. *J Pathol.* 2004; **204**: 121 – 130.
- 17 Li SS, Xue WC, Khoo US, Ngan HY, Chan KY, Tam IY, et al. Replicative MCM7 proteins as a proliferation marker in endometrial carcinoma: a tissue microarray and clinicopathological analysis. *Histopathology.* 2005; **46**: 307 – 313.
- 18 Guida T, Salvatore G, Faviana P, Gianinni R, Garcia-Rostan G, Provitera L, et al. Mitogenic effects of the up-regulation of minichromosome maintenance (MCM) proteins in anaplastic thyroid carcinoma. *J Clin Endocrinol Metab.* 2005; **190**: 4703 – 4709.
- 19 Chatrah P, Scott IS, Morris LS, Davies RJ, Rushbrook SM, Bird K, et al. Aberrant expression of minichromosome maintenance protein 2 and Ki-67 in laryngeal squamous epithelial lesions. *Br J Cancer.* 2003; **89**: 1048 – 1054.
- 20 Davidson EJ, Morris LS, Scott IS, Rushbrook SM, Bird K, Laskey RA, et al. Minichromosome maintenance (Mcm) proteins, cyclin B1 and D1, phosphohistone H3 and in situ DNA replication for functional analysis of vulval intraepithelial neoplasia. *Br J Cancer.* 2003; **88**: 257 – 262.
- 21 Scott IS, Heath TM, Morris LS, Rushbrook SM, Bird K, Vowler SL, et al. A novel immunohistochemical method for estimating cell cycle phase distribution in ovarian serous neoplasms: implications for the histopathological assessment of paraffin-embedded specimens. *Br J Cancer.* 2004; **90**: 1583 – 1590.
- 22 Lukes RJ, Craver LF, Hall TC, Rappaport H, Ruben R. Report of the Nomenclature Committee. *Cancer Res.* 1966; **26**: 1311.
- 23 Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. The World Health

- Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. Report of the Clinical Advisory Committee meeting, Airlie House, Virginia, November, 1997. *Ann Oncol.* 1999; **10**: 1419 – 1432.
- 24 Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem.* 1981; **29**: 577 – 580.
  - 25 Garcia JF, Camacho FI, Morente M, Fraga M, Montalban C, Alvaro T, et al. Hodgkin and Reed-Sternberg cells harbor alterations in the major tumor suppressor pathways and cell-cycle checkpoints: analyses using tissue microarrays. *Blood.* 2003; **101**: 681 – 689.
  - 26 Morente MM, Piris MA, Abaira V, Acevedo A, Aguilera B, Bellas C, et al. Adverse clinical outcome in Hodgkin's disease is associated with loss of retinoblastoma protein expression, high Ki-67 proliferation index, and absence of Epstein-Barr virus-latent membrane protein 1 expression. *Blood.* 1997; **90**: 2429 – 2436.
  - 27 Gerdes J, van Baarlen J, Pileri S, Schwarting R, Vanunnik JA, Stein H. Tumor cell growth fraction in Hodgkin's disease. *Am J Pathol.* 1987; **128**: 390 – 393.
  - 28 Claviez A, Tiemann M, Peters J, Kreipe H, Schneppenheim R, Parwaresch R. The impact of EBV, proliferation rate, and Bcl-2 expression in Hodgkin's disease in childhood. *Ann Hematol.* 1994; **68**: 61 – 66.
  - 29 Morgan KG, Quirke P, O'Brien CJ, Bird CC. Hodgkin's disease: a flowcytometric study. *J Clin Pathol.* 1988; **41**: 365 – 369.
  - 30 Joensuu H, Klemi PJ, Korkeila E. Prognostic value of DNA ploidy and proliferative activity in Hodgkin's disease. *Am J Clin Pathol.* 1988; **90**: 670 – 673.
  - 31 Erdkamp FL, Breed WP, Schouten HC, Janssen WC, Hoffmann JJ, Wijnen JT, et al. DNA aneuploidy and cell proliferation in relation to histology and prognosis in patients with Hodgkin's disease. *Ann Oncol.* 1993; **4**: 75 – 80.
  - 32 Bai M, Tsanou E, Agnantis NJ, Kamina S, Grepic C, Stefanaki K, et al. Proliferation profile of classical Hodgkin's lymphomas. Increased expression of the protein cyclin D2 in Hodgkin's and Reed-Sternberg cells. *Mod Pathol.* 2004; **17**: 1338 – 1345.
  - 33 Tiemann M, Claviez A, Lüders H, Zimmermann M, Schellong G, Dörffel W, et al. Proliferation characteristics in pediatric Hodgkin's lymphoma point to a cell cycle arrest in the G(1) phase. *Mod Pathol.* 2005; **18**: 1440 – 1447.
  - 34 Brake T, Connor JP, Petereit DG, Lambert PF. Comparative analysis of cervical cancer in women and in a human papillomavirus-transgenic mouse model: identification of minichromosome maintenance protein 7 as an informative biomarker for human cervical cancer. *Cancer Res.* 2003; **63**: 8173 – 8180.
  - 35 Heidebrecht HJ, Buck F, Steinmann J, Sprenger R, Wacker HH, Parwaresch R. p100: a novel proliferation-associated nuclear protein specifically restricted to cell cycle phases S, G2, and M. *Blood.* 1997; **90**: 226 – 233.
  - 36 Schrader C, Janssen D, Klapper W, Siebmann JU, Meusers P, Brittinger G, et al. Minichromosome maintenance protein 6, a proliferation marker superior to Ki-67 and independent predictor of survival in patients with mantle cell lymphoma. *Br J Cancer.* 2005; **93**: 939 – 945.
  - 37 Schrader C, Janssen D, Meusers P, Brittinger G, Siebmann JU, Parwaresch R, et al. Repp86: a new prognostic marker in mantle cell lymphoma. *Eur J Haematol.* 2005; **75**: 498 – 504.
  - 38 Obermann EC, Eward KL, Dogan A, Paul EA, Loddo M, Munson P, et al. DNA replication licensing in peripheral B-cell lymphoma. *J Pathol.* 2005; **205**: 318 – 328.
  - 39 Drexler HG, Gignac SM, Hoffbrand AV, Minowada J. Formation of multinucleated cells in a Hodgkin's-disease-derived cell line. *Int J Cancer.* 1989; **43**: 1083 – 1090.
  - 40 Gupta RK, Lister TA, Bodmer JG. Proliferation of Reed-Sternberg cells and variants in Hodgkin's disease. *Ann Oncol.* 1994; **5** (suppl 1): 117 – 119.
  - 41 Schlegelberger B, Weber-Matthiesen K, Himmler A, Bartels H, Somen R, Kuse R, et al. Cytogenetic findings and results of combined immunophenotyping and karyotyping in Hodgkin's disease. *Leukemia.* 1994; **8**: 72 – 80.
  - 42 Falzetti D, Crescenzi B, Matteuci C, Falini B, Martelli MF, van Den Berghe H, et al. Genomic instability and recurrent breakpoints are main cytogenetic findings in Hodgkin's disease. *Haematologica.* 1999; **84**: 298 – 305.