Immunohistochemical Expression and Prognostic Significance of CCND3, MCM2 and MCM7 in Hodgkin Lymhoma

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Abstract. *Background: Increased proliferation rate may be associated with inferior outcome in patients with Hodgkin lymphoma (HL). Minichromosome maintenance proteins (MCMs) and D–type cyclins are essential for DNA replication. Patients and Methods: Lymph node sections from 138 HL patients were immunohistochemically stained for cyclin D3 (CCND3), MCM2 and MCM7 aiming to investigate clinical outcome. Results: Higher MCM2 expression was observed in patients in early stage disease and normal albumin levels; higher MCM7 was found for asymptomatic patients, early stage disease, <5 involved sites, no anemia and normal albumin levels; higher CCND3 expression was found for older patients and normal lactate dehydrogenase (LDH). Univariate analysis revealed no correlation with failure-free (FFS) or overall survival (OS). Multivariate analysis revealed that high MCM7 expression was an adverse prognostic factor for OS, along with older age and advanced stage, while it was of borderline significance for FFS when adjusted for stage. Conclusion: These results suggest that MCM7 deserves further evaluation as a potential independent prognostic factor in larger patient series.*

Despite significant advances in the treatment of Hodgkin lymphoma (HL), complete remission is not achieved or a relapse occurs in 25-30% of patients after first–line treatment with adriamycin, bleomycin, vinblastine and dacarbazine

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(ABVD) or equivalent chemotherapy (CT), with or without radiotherapy (RT). Therefore, a considerable proportion of patients, especially those who present in advanced stages, could be candidates for more aggressive first–line treatment, such as increased–dose BEACOPP chemotherapy, which provides improved tumor control and overall survival at the cost, however, of increased hematologic toxicity, toxic deaths and late toxicity (1). However, the pre–treatment recognition of patients with poor prognosis is difficult, since none of the major research groups has been able to reproducibly determine sizeable subgroups of patients with >50% probability of failure (2-4). On the other hand, many patients treated with less chemotherapy according to response-adapted strategies have been cured (2), suggesting possible overtreatment of many early-stage, as well as of up to 30% of advanced-stage patients with current standard chemotherapy protocols. The identification of low-risk patients that could eventually benefit from treatment reduction is also of great importance, given that long term complications may affect survival of otherwise cured patients with HL.

Accumulated evidence suggests that Hodgkin Reed–Sternberg (HRS) cells, the neoplastic cells of classical HL, are characterized by severely impaired cell cycle regulation and apoptosis (5-8). Many cell cycle regulating proteins, such as cyclins D1, D2, D3, E1, E2, B1, cyclin-dependent kinases (CDKs) 1, 2 and 6, p21[CIP1], P27[KIP1], p53, retinoblastoma protein (pRB), proliferating cell nuclear antigen (PCNA) and minichromosome maintenance protein 6 (MCM6) are abnormally expressed in HL (5, 7, 9, 10). Cyclins and CDKs are key molecules forming the cyclin-CDK complexes, which regulate cell cycle progression. Levels of CDKs remain stable throughout the entire cell cycle, whereas the levels of cyclins vary, depending on cell cycle progression. Cyclins are classified into two main functional families. The G_1 family includes cyclins D1, D2, D3 and E, while the other family includes cyclins A, B1 and

B2. The G_1-family cyclins are essential for the passage of cells through the G_1–phase and entry into the S–phase. The cyclin D (CCND)–CDK4 and CCND–CDK6 complexes regulate the transition from G_0 to G_1, being necessary for the full activity of the E2F–transcription factor family through the inhibition of pRB, while the CCNE–CDK2 complex regulates the transition of cells from G_1 to S (11).

Minichromosome maintenance proteins drive the formation of prereplicative complexes (PRCs), which is the first key event during the G_1 phase. There is a large body of evidence demonstrating that the transition from the cell cycle to quiescence (G_0 phase) is linked to the down-regulation of the MCM2–7 protein complexes. In all eukaryotic cells, initiation of DNA synthesis is a complex multistep process tightly coupled to progression through the cell cycle. The heterohexamer MCM2–7 complexes function as replicative helicases; complexes are present in the nucleus throughout the whole cell cycle and bind to the PRCs at late G_1 phase. The initiation of DNA replication depends on the formation of the PRCs at the replication origins (RO) at early G_1 phase, as well as at late mitosis (12-14).

Evaluation of cell cycle regulators has gained special interest in the effort to increase the amount of prognostic information in malignant tumors (15). The increased expression of proliferation markers, including Ki-67, PCNA, CCNA and others, has been associated with poor prognosis in various types of malignant tumor (16). The same applies to the immunohistochemical expression of MCMs (14, 15, 17-19).

In HL however, none of the proliferation markers evaluated so far has been reproducibly associated with prognosis, with the exception of topoisomerase IIα, the increased expression of which is an adverse prognostic factor (20–25). In the present study, we evaluated the expression of three molecules involved in the cell cycle and cell proliferation, namely CCND3, MCM2 and MCM7, as well as the established proliferation marker Ki-67, in the HRS cells of a relatively large series of patients with HL, aiming to assess their correlation with baseline clinical, laboratory and pathologic parameters and clinical outcome.

Patients and Methods

Patients and staging. We retrospectively analyzed 138 non-pediatric, HIV-negative patients with histologically proven HL, for whom paraffin-embedded lymph node tissue was available at diagnosis between 1988 and 2003. The vast majority of the patients (129/138; 93%) had received treatment with anthracycline-based CT with or without RT . All histologic material was reviewed and classified according to the recent WHO classification (26). All patients were clinically staged according to the Ann–Arbor system, using standard staging procedures; clinical stages IA and IIA were considered early, while clinical stages IB, IIB, III and IV were considered advanced; age, leukocytosis, severe lymphocytopenia and serum albumin levels were analyzed at the International Prognostic Score

(IPS) cut-offs of ≥45 years, ≥15×10⁹/l, <0.6×10⁹/l or <8% and <4 g/dl respectively (2, 3, 27). The number of involved anatomic sites was categorized as <5 and ≥5 (3). Anemia was defined as hemoglobin levels <13 g/dl for males and <11.5 g/dl for females (27) and the cut-off for erythrocyte sedimentation rate (ESR) was set at 50 mm the first hour. Lactate dehydrogenase (LDH) levels were analyzed as normal *vs.* abnormal with reference to the upper normal limit of the laboratory.

Treatment strategies. Treatment strategies for early Ann–Arbor stage (AAS IA, IIA) and advanced stage (IB, IIB, III, IV) patients have been described previously (27). Early-stage patients were scheduled for combined modality therapy including low-dose involved field RT. Advanced-stage patients received 6-8 cycles of CT, but additional RT was given to selected patients. Of the total 138 patients, 129 received CT with ABVD or equivalent regimens with or without RT; 8 received nitrogen mustard, vincristine, procarbazine and prednisone (MOPP) or similar regimens such as chlorambucil, vinblastine, procarbazine and prednisone (ChIVPP) with or without RT; 1 patient was treated with RT only. Of the total 138 patients, 104 (75%) received RT, of whom 92 (88%) involved field RT (IFRT); 29 patients (21%) did not receive RT; for 5 (4%) patients no data regarding RT could be retrieved.

Immunohistochemical staining. All tissue samples were fixed in 10% buffered formalin solution and embedded in paraffin. Four micrometer serial sections were cut from each specimen on superfrost plus glass slides and left to dry overnight at 37°C. Monoclonal antibodies were used against CCND3 (Clone DCS–22; applied at a dilution 1:100 overnight), MCM2 (Clone CRCT22.1; applied at a dilution 1:100 overnight), MCM7 (Clone DCS–141.1; applied at a dilution 1:50 overnight), all from Novocastra/Leica Microsystems Inc., Bannockburn, USA; and Ki-67 (Clone MIB–1; applied at a dilution 1:50 overnight, Dako Denmark A/S Glostrup, Denmark).

Prior to immunohistochemical staining against antibodies CCND3, MCM2 and MCM7, all sections were incubated four times for 5 min in citrate buffer pH 6.0, at 750 W, in a microwave oven; prior to immunohistochemical staining against Ki-67 all sections were incubated four times for 5 min in citrate buffer pH 9.0, at 750 W, in a microwave oven. Application of the primary antibody was followed by the two-step horseradish peroxidase technique, using Dako Envision Kit (Dako, Carpinteria, CA, USA). All specimens were treated using identical procedures. Negative controls (sections in which the primary antibody was substituted with nonimmune mouse serum) were also stained in each run. External controls consisted of tonsillar tissue, whereas the residual germinal centers seen in most cases served as internal positive controls to ensure the adequacy of immunohistochemical staining.

Staining evaluation and image analysis. All immunohistochemically stained slides that fulfilled the internal as well as external positive and negative controls were evaluated. Any HRS nuclear staining for antibodies CCND3, MCM2, MCM7 and Ki-67 was considered as positive, while weak cytoplasmic staining, when observed, was considered as non-specific and was not taken into account. All immunohistochemically stained slides for CCND3, MCM2, MCM7 and Ki-67 were scanned, field by field, at ×10, ×20 and ×40 magnification to identify regions of maximum HRS cell density (hot spot) by two observers (AM and EB) without knowledge of the patients' clinical data. The hot spots were marked. Given that the vast

majority of the cell population in HL consists of non-neoplastic cells, our goal was to include at least 50-100 HRS cells during the evaluation of each case. In those cases, in which HRS cells could not be directly distinguished from their inflammatory environment, nuclear morphology from the corresponding hematoxylin/eosin (H&E) slides and/or CD30 immunohistochemical staining were used. When consensus was reached with regard to the number of hot spots that would be photographed from each case, the magnification was changed to ×20, the hot spots were photographed (Zeiss Axiolab Microscope, Carl Zeiss Jena, GmBH Jena, Germany) and photographs were digitalized and stored as 24–bit BMP files. In the vast majority of cases, two photographs per case were enough, but there were a few cases in which, although 10 photographs were taken, fewer than 50 HRS cells could be identified. For the quantitative assessment of the digital images, two Image Analysis Software programs were used (Image Sigma Scan Software, Jandel Scientific, Ekrath, Germany, and Color Estimator, Version 2.2.1, 2003), Petros M Pavlopoulos, MD, Department of Pathology, Medical School, National and Kapodistrian University of Athens, Greece). Briefly, with both programs, the operator marked all the HRS cells in each digital image and obtained the result in a digital format (*i.e.* number and percentage of immunohistochemically stained HRS cells *vs.* non-stained HRS cells).

Statistical analysis. The percentages of HRS cells expressing CCND3, MCM2, MCM7 and Ki-67 in various subgroups of patients defined by their baseline characteristics were compared by the Mann-Whitney *U*-test or the Kruskal–Wallis test, as appropriate. The correlation between CCND3, MCM2, MCM7 and Ki-67 expression was evaluated by Spearman's rho coefficient. Failure–free survival (FFS) was defined as the time interval between treatment initiation and treatment failure or last follow-up. Treatment failure was defined as inability to achieve complete (CR) or partial remission (PR) during initial therapy requiring a switch to another CT regimen, death during initial therapy, or progression after an initial CR or PR. Overall survival (OS) was defined as the time interval between treatment initiation and death of any cause or last follow–up. Survival curves were plotted by the method of Kaplan–Meier. The identification of prognostic factors in univariate analysis was based on the log-rank test. Multivariate analysis was performed using Cox's proportional hazards model. A stepwise selection procedure, with entry and removal criteria of $p=0.05$ and $p=0.10$, respectively, was used. In order to avoid the use of arbitrary cutoffs, the main survival analysis was performed by treating proliferation markers as continuous covariates.

Results

Patients' characteristics. Patients' clinical and laboratory characteristics were compatible with other reported unselected series of patients with non-pediatric HL. The median age of the 138 patients was 31.5 (range, 15-78) years and 76 (55%) were males. 134 cases were classified as classical HL (97%) and 4 cases as nodular lymphocyte predominance (NLP) HL (3%). In detail, 93 patients were classified as having disease of nodular sclerosis (NS) subtype (67%), 34 cases as mixed cellularity (MC) (25%), 5 as lymphocyte rich (4%), 1 as lymphocyte depletion (<1%) and 1 as classical HL, unclassified (<1%). Clinical stage was I,

II, III and IV in 41 (29%), 62 (45%), 20 (15%) and 15 (11%) of patients, respectively, while 47 (34%) had B-symptoms. Overall, 58/138 patients (42%) had advanced-stage disease (stage IB, IIB, III, IV) and 19 (14%) had 5 or more anatomic sites involved. Anemia was recorded in 48/136 patients (35%), leukocytosis $\geq15\times10^9$/l in 16/136 (12%), severe lymphocytopenia in 9/122 (7%), low albumin levels in 33/108 (31%), elevated ESR ≥50 mm/h in 44/104 (42%) and elevated LDH in 31/102 (30%) of patients. The median follow-up of patients who were alive at the time of the analysis was 109 (29-257) months.

Evaluation of proliferation marker expression. The immuno-histochemical expression of CCND3 was evaluated in 113 patients. Evidence of CCND3 expression by the HRS cells was present in 105/113 (93%). The pattern of staining was diffuse nuclear; faint cytoplasmic staining was seen in some cases which was disregarded as nonspecific (Figure 1a). Furthermore, immunohistochemical expression of CCND3 was seen in all cases in larger cells within residual germinal centers. The median percentage of CCND3-positive HRS cells was 24% (interquartile range, IQR, 12-47%) and the range was 2% to 98% in cases with identifiable CCND3 expression.

MCM2 and MCM7 expression was evaluated in 116 and 121 patients, respectively. The pattern of staining was mostly nuclear for both MCM2 and MCM7; similarly to CCND3, faint cytoplasmic staining was seen in some cases, which was disregarded as non-specific (Figures 1b and 1c). MCM2 and MCM7 expression was also seen in all cases in larger cells within residual germinal centers. Evidence of MCM2 expression in the HRS cells was present in 115/116 (99%); the median percentage of MCM2-positive HRS cells was 63% (IQR, 38-81%) and the range was 7% to 99% in cases with identifiable MCM2 expression. Evidence of MCM7 expression in the HRS cells was present in all cases; the percentage of MCM7-positive HRS cells ranged from 15% to 100%, with a median value of 88% (IQR, 81-94%).

Ki-67 expression was evaluated in 95 patients. The staining pattern was diffuse nuclear. All cases were Ki-67-positive, with the percentage of positive HRS cells ranging from 16% to 100%, with a median value of 77% (IQR, 57-88%).

Correlation between proliferation markers' expression and patients' characteristics. A statistically significant correlation was found between MCM2 and MCM7 expression (Spearman's rho 0.279, $p=0.004$). There was a marginally significant negative correlation between MCM2 and Ki-67 expression (Spearman's rho –0.206, $p=0.06$), but no evidence of correlation between MCM7 and Ki-67 expression (Spearman's rho 0.061, $p=0.58$). There was also no correlation between CCND3 expression and that of MCM2, MCM7 or Ki-67 (Spearman's rho 0.005, $p=0.96$; 0.15, $p=0.14$; and 0.038, $p=0.74$, respectively).
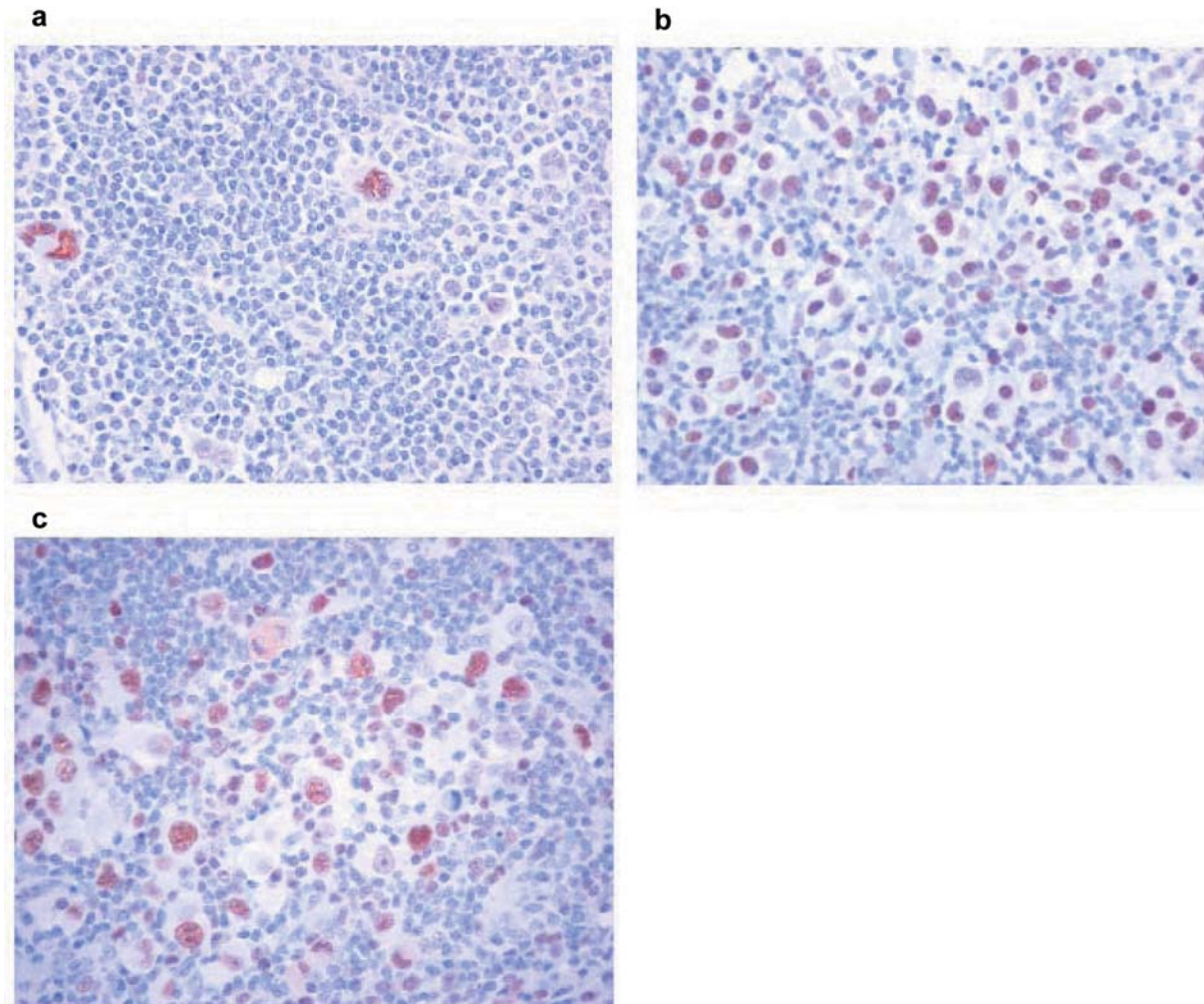
Figure 1. *Representative examples of (a) CCND3, (b) MCM2 and (c) MCM7 expression in patients with Hodgkin lymphoma.*

As shown in Table I, higher CCND3 expression was associated with older age (p=0.03) and normal LDH levels (p=0.05). Higher MCM2 expression was associated with earlier clinical stage (p=0.03) and normal albumin levels (>4 g/dl, p=0.002). Higher MCM7 expression was associated with earlier clinical stage (p=0.005), absence of B-symptoms (p=0.004), lower number of involved anatomic sites (p=0.009), absence of anemia (p=0.02) and normal albumin levels (>4 g/dl, p=0.005). Higher Ki-67 expression was only associated with a higher number of involved anatomic sites (p=0.03).

Univariate analysis. Among the 138 patients, 31 failures accounted for a 5- and 10-year FFS of 80% and 77% respectively. Furthermore, 25 patients had died at the time of the analysis, giving a 5- and 10-year OS of 88% and 85%. Many of the established conventional prognostic factors

listed in Table I emerged as adverse predictors of FFS, namely advanced stage, B-symptoms, involvement of 5 or more anatomic sites, severe lymphocytopenia, low serum albumin levels and IPS ≥3 (data not shown). The same parameters (albumin was of borderline significance), as well as age ≥45 years old, were also predictive of OS in univariate analysis (data not shown). An exploratory analysis was performed in which CCND3, MCM2 and MCM7 expression were evaluated at various cut-offs (10, 20, 30, 40, 50 and 60% for CCND3; 30, 40, 50, 60, 70, 80 and 90% for MCM2; 80, 85, 90 and 95% for MCM7). None of these comparisons provided any significant difference in terms of FFS and OS regarding CCND3, MCM2 or MCM7 expression.

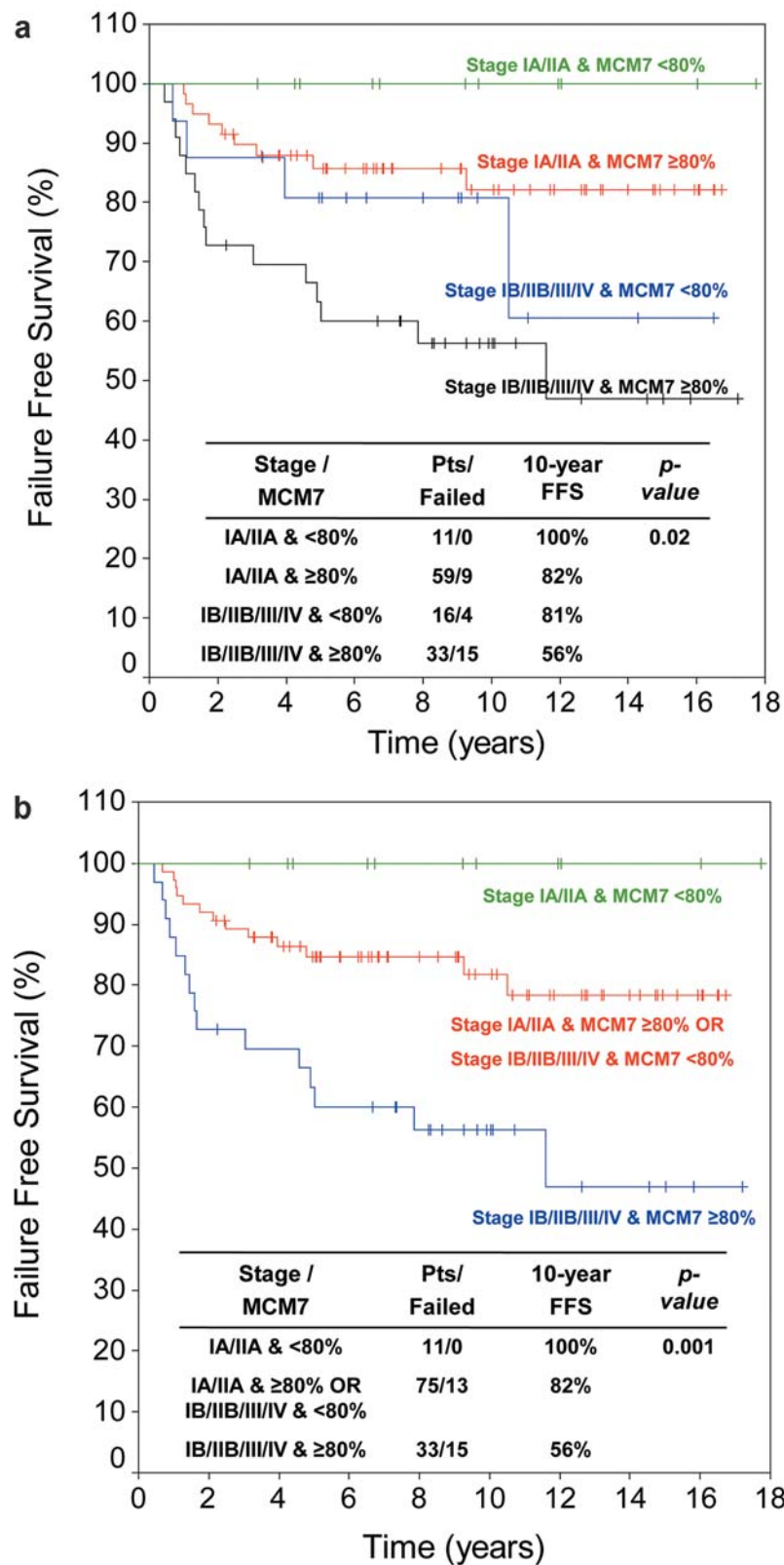*Multivariate analysis.* Although univariate analysis failed to reveal any association between CCND3, MCM2 or MCM7

Figure 2. *Failure free survival according to clinical stage and MCM7 expression by HRS cells. a: All four possible categories are shown. b: The same data with the two intermediate groups merged [i.e. early stage, high MCM7 (≥80%) and advanced stage, low MCM7 (<80%)].*

Table I. *Correlation between proliferation marker expression and baseline patient characteristics.*

Patient characteristics	CCND3 (%)	MCM2 (%)	MCM7 (%)	Ki-67 (%)
Age	23.3 *vs.* 32.0	63.1 *vs.* 63.7	88.4 *vs.* 87.0	75.5 *vs.* 79.5
(<45 *vs.* ≥45 years)	*p*=0.03	NS *	NS	NS
Gender	30.8 *vs.* 18.8	62.4 *vs.* 67.9	89.2 *vs.* 87.8	77.1 *vs.* 76.8
(males *vs.* females)	NS	NS	NS	NS
Clinical Stage	25.6 *vs.* 23.1	70.3 *vs.* 59.4	90.2 *vs.* 84.9	74.2 *vs.* 78.1
(early *vs.* advanced)	NS	*p*=0.03	*p*=0.005	NS
B–symptoms	30.4 *vs.* 17.5	66.7 *vs.* 59.8	89.7 *vs.* 84.3	78.5 *vs.* 76.5
(absent *vs.* present)	NS	NS	*p*=0.004	NS
Involved sites (#)	25.5 *vs.* 16.0	66.0 *vs.* 59.3	88.7 *vs.* 78.6	76.5 *vs.* 89.1
(<5 *vs.* ≥5)	NS	NS	*p*=0.009	*p*=0.03
Anemia	29.9 *vs.* 17.4	65.8 *vs.* 62.5	89.7 *vs.* 85.8	77.4 *vs.* 76.8
(no *vs.* yes)	NS	NS	*p*=0.02	NS
Leukocyte counts	24.4 *vs.* 14.8	66.0 *vs.* 39.0	87.7 *vs.* 88.2	76.8 *vs.* 83.3
(<15 *vs.* ≥15×10^9/L)	NS	NS	NS	NS
Lymphocytopenia	24.9 *vs.* 14.5	63.6 *vs.* 69.8	87.7 *vs.* 94.4	76.8 *vs.* 87.8
(no *vs.* yes)	NS	NS	NS	NS
ESR (mm/h)	33.3 *vs.* 19.0	49.1 *vs.* 69.4	90.4 *vs.* 87.0	82.5 *vs.* 74.2
(<30 *vs.* ≥30)	NS	NS	NS	NS
Serum Albumin	27.3 *vs.* 15.4	73.1 *vs.* 47.1	90.9 *vs.* 82.1	79.5 *vs.* 79.7
(≥4 *vs.* <4 g/dL)	NS	*p*=0.002	*p*=0.005	NS
Serum LDH	25.5 *vs.* 15.4	67.4 *vs.* 63.6	87.7 *vs.* 88.7	76.7 *vs.* 74.4
(normal *vs.* elevated)	*p*=0.05	NS	NS	NS
Histology	23.8 *vs.* 19.0	63.1 *vs.* 65.5	88.9 *vs.* 85.8	77.4 *vs.* 77.1
(Nod** *vs.* MC***)	NS	NS	NS	NS
IPS⁋ (<3 *vs.* ≥3)	25.5 *vs.* 16.0	70.4 *vs.* 59.3	88.6 *vs.* 80.9	78.5 *vs.* 76.8
	NS	NS	NS	NS

*NS=Non–Significant, **Nod=Nodular Sclerosis, ***MC=Mixed Cellularity, ⁋IPS=International Prognostic Score.

expression and FFS at any cut-off level, we were somewhat concerned regarding the possibility that a potential adverse impact of MCM2 or MCM7 on FFS could have been overshadowed by their inverse association with other well-known adverse prognostic factors. This was especially relevant for MCM7 expression, which was inversely correlated with many of the examined adverse prognostic factors, *i.e.* lower MCM7 expression was found in patients with advanced-stage disease, B-symptoms, involvement of ≥5 anatomic sites, anemia and low serum albumin levels (Table I).

Indeed, when MCM7 expression was evaluated as a continuous covariate along with advanced stage and the number of involved sites (Table II), its impact on FFS was nearly significant: any increase in MCM7 expression by 1% resulted in an increase in the hazard ratio for failure of 1.038 (95% confidence interval, CI=0.998-1.080, *p*=0.061). This result of borderline significance was also evident when MCM7 expression was dichotomized at the cut-off of 80% (hazard ratio=2.64, 95% CI=0.90-7.72, *p*=0.076), but not at the cut-offs of 85%, 90% or 95% (*p*>0.10).

In multivariate analysis of overall survival, when MCM7 expression was evaluated as a continuous covariate along with advanced clinical stage and older age (Table III), its impact on OS became statistically significant: any increase

in MCM7 expression by 1% resulted in an increase in the hazard ratio for death of 1.047 (95% confidence interval 1.001-1.094, *p*=0.045). This statistically significant result was also evident when MCM7 expression was dichotomized at the cut-offs of 85% (hazard ratio=2.79, 95% CI=1.05–7.42, *p*=0.04) and 95% (hazard ratio=4.13, 95% CI=1.36-12.52, *p*=0.01). At the cut-off of 90%, the hazard ratio for death was 2.13 (95% CI=0.093-4.91, *p*=0.075); no statistical significance was seen at the cut-off of 80% (*p*>0.10). In contrast to MCM7, MCM2 and CCND3 had no independent effect on FFS or OS in multivariate analysis.

The combined effect of clinical stage and MCM7 expression on the outcome of patients with HL is schematically provided in Figures 2a and 2b. Patients with early-stage disease with MCM7 expression in <80% of HRS cells had an excellent outcome, with no failures among 11 patients, while those with ≥80% MCM7 expression had a 10–year FFS of 82%; the difference was marginally significant (*p*=0.18; Figure 2a). Patients with advanced-stage disease with low MCM7 expression (<80%) had a 10–year FFS of 81%, while the corresponding figure for those with high MCM7 expression was 56% (*p*=0.23; Figure 2a); the latter group included 28% of the whole population of patients with HL, but also 67% of the population of patients

Table II. *Multivariate analysis of failure free survival: MCM7 expression (continuous covariate), clinical stage and the number of involved anatomic sites were evaluated as potential independent prognostic factors.*

Prognostic factor	Relative risk of failure		
	RR	95% CI	*p*-value
Advanced stage (IB/IIB/III/IV *vs*. IA/IIA)	4.37	1.93-9.87	<0.001
MCM7 (per 1% increase)	1.038	0.998-1.080	0.061
Involved anatomic sites (≥5 *vs*. <5)		NS	

RR, Relative risk; CI, confidence intervals; NS, non-significant.

Table III. *Multivariate analysis of overall survival: MCM7 expression (continuous covariate), clinical stage and age were evaluated as potential independent prognostic factors.*

Prognostic factor	Relative risk of death		
	RR	95% CI	*p*-value
Advanced stage (IB/IIB/III/IV *vs*. IA/IIA)	4.83	1.90-12.32	0.001
Age (≥45 *vs*. <45 years)	5.24	2.20-12.48	<0.001
MCM7 (per 1% increase)	1.047	1.001-1.094	0.045

RR, Relative risk; CI, confidence intervals.

with advanced disease. Since the outcome of patients with early stage disease and high MCM7 expression or advanced stage disease and low MCM7 expression was very similar, we also merged these subgroups, as shown in Figure 2b: this combined group included patients with a 10-year FFS of 82%, which was significantly superior to that of the advanced-stage/high MCM7 subgroup (p=0.002) and tended to be inferior to that of the early-stage/low MCM7 subgroup (p=0.15).

Discussion

The neoplastic cells of HL, the HRS cells, appear to be actively proliferating cells that undergo unsuccessful cell cycles and fail to multiply due to their inability to divide correctly, thus resulting in polyploidy (16). Although most researchers appear to conclude that the majority of HRS cells are arrested in the G_1-phase (7, 9, 10, 28), others have reported that 80-90% of HRS cells are actually in S- or G_2-phase, with only 10-20% being in G_1, and that the overexpression of cyclins of the G_1 family does not reflect proliferation but rather reduced proteolytic degradation (16). The expression and prognostic significance of cyclins have rather recently been evaluated in HL (6, 7, 9, 22, 23, 29): CCNA, B1 and E are expressed in the vast majority of cases (29), while, according to a recent study, CCNA2 and CCNE2 may be implicated in the prognosis of the disease (22). On

the other hand, D–cyclins are less frequently expressed (7, 9, 16, 29) and did not present any association with prognosis in a single study (29).

In our analysis, we observed a median CCND3 expression of 24% (range 2-98%), while CCND3 expression was not seen in 7% of cases. This observation is in agreement with previous studies reporting that CCND3 overexpression by HRS cells is not so frequent (6, 7, 9). Additionally, our data provide a clearer view as far as CCND3 expression in classical HL is concerned; Tzankov *et al*. (7) initially reported that CCND3 was detectable in 58% (146/253) of their classical HL cases, but did not provide the percentage of expression in HRS cells in a later study in which they stated that increased G_1-phase cyclin levels probably reflect lower proteolysis rather than true proliferation (16); Bai *et al*. did not report the percentage of CCND3 expression in a series of 103 cases (9), as the data provided refer to "39% CCND3 overexpression", defined as >10% CCND3 expression by HRS cells in 10 optical fields with x40 magnification. Using the same cut-off, Montalbán *et al*. reported that CCND3 was overexpressed in 41.7% of patients with HL (29).

To the best of our knowledge, the present study is the first one evaluating the expression of MCM2 and MCM7 and their potential prognostic significance in HL: MCM2 expression was observed at a median of 63% of HRS cells (range 7-99%) and MCM7 at a median 88% (range 15-100%), while no statistically significant differences were

found concerning the major histologic HL subtypes (nodular sclerosing and mixed cellularity). Furthermore, we observed a positive, albeit not very strong, correlation between MCM2 and MCM7 expression. This correlation probably reflects the similar kinetics of the two proteins, as reported for other neoplasms as well, namely their up-regulation during cell proliferation and tumor development and down-regulation at cell cycle exit (12, 15, 30).

The higher MCM7 expression compared to Ki-67 (median 88% *vs.* 77%) is in agreement with previously published observations in dysplastic conditions (*e.g.* dysplasia in cervical squamous neoplasia and bladder urothelial neoplasia), as well as in muscle-invasive urothelial cancer (15). Additionally, this observed difference is in agreement with that the majority of HRS cells, as most researchers appear to conclude, are arrested in the G_1-phase (7, 9, 10, 28), as well as MCMs being expressed throughout all replicative phases, while Ki-67 – the role of which is not yet clearly understood – may not be expressed until late G_1-phase (31, 32).

The expression of MCM6 by HRS cells has been recently immunohistochemically evaluated in a series of 55 patients with classical HL (10). The authors reported high MCM6 expression (median 85%, range 35-99%) without significant differences among histologic subtypes. These findings, coupled by the fact that we found both MCM2 and MCM7 to be overexpressed, are in agreement with the MCM2–7 heterohexamer function in the G_1-phase (14).

The different median percentage of MCM2 and MCM7 expression by HRS cells reported here should not be regarded as problematic; one should take into account the hypothesis that MCMs may serve other functions (33). This hypothesis is consistent with the observation that the members of the MCM family undergo several post–transcriptional changes, including phosphorylation, polyubiquitination and acetylation, as well as the fact that MCM2–7 members are found and may function individually, and not only as MCM2–7 heterohexamers, at places other than the ROs in the genome (34, 35). Additionally, a recent proteomic study, which examined the individual expression of the MCM2–7 family members in meningiomas compared to normal arachnoideal tissue, demonstrated that various MCM molecules may not be overexpressed to the same degree in meningiomas: MCM2 octuples, MCM3 quintuples, MCM4 and MCM5 quadruple, MCM6 triples and MCM7 quintuples (34), thus indicating, as our results do, that although MCMs act as heterohexamers and have similar kinetics in most cases, it should not necessarily be expected that their individual expression in tumors would increase in parallel.

Higher MCM2 expression correlated with earlier stages and serum albumin levels >4 g/dl. However, the most powerful and convincing correlations with established prognostic factors for HL were observed for MCM7; it was inversely correlated with advanced stage disease, B-symptoms, ≥5 involved anatomic sites, anemia and lower serum albumin levels. This inverse correlation of lower MCM expression with adverse prognostic factors is not without precedence, since it has recently been reported in colorectal carcinoma, where the labeling index for both MCM2 and MCM7 expression was lower in Dukes' stage C tumors than in Dukes' stage B ones but, paradoxically, a high index for MCM7 was shown to be an independent prognostic factor, while that for MCM2 was not (36).

In our study, no independent prognostic value of MCM7 expression with respect to FFS or OS was found in univariate analysis. However, we were concerned that any eventual adverse prognostic impact of MCM7 expression could have been masked by its inverse relationship with other known prognostic factors. Indeed, when analyzed in multivariate models along with advanced-stage disease, MCM7 expression emerged as a statistically significant independent prognostic factor for OS (after further control for age), as well as being a marginally significant independent predictor of FFS. Our data generate the hypothesis that high MCM7 expression by the HRS cells might represent a novel prognostic factor in HL. Since the majority of patients (67%) with advanced HL have high MCM7 expression, this might represent a potential advantage because the incorporation of MCM7 might facilitate the identification of sizeable subgroups of patients with sufficiently poor outcome, suitable for experimental treatment approaches. However, under strict interpretation with the given sample size, we have only shown that higher MCM7 expression provides prognostic information that is independent of disease stage, number of involved sites and patient age. Whether the impact of MCM7 expression is also independent from other well-established conventional prognostic factors or even biological markers needs to be investigated in larger studies. Further studies should also clarify a reasonable cut-off for MCM7 expression to be adopted in clinical practice, should its independent prognostic significance be prospectively validated.

According to our findings, HL may be added to the list of malignant neoplasms in which the immunohistochemical expression of MCMs affects clinical outcome; mainly MCM2 and MCM5 and less frequently MCM7 have been reported to do so in breast cancer, brain tumors, non-small cell lung cancer, prostate cancer, urinary bladder cancer, esophageal cancer, renal cell carcinomas and oral cavity squamous cell carcinoma (14, 15, 17-19). High labeling index for MCM7, has recently been shown to be related to poor prognosis in patients with colorectal carcinoma (36), as well as stage I lung adenocarcinoma (37). Interestingly, in the latter cases, MCM2 was not informative as a prognostic indicator (36, 37). This discrepancy, also observed for HL in the present study, underlines the distinct biologic/prognostic role that MCM proteins might play.

In contrast to MCM7, the expression of MCM2, CCND3 and Ki-67 was not associated with the outcome of patients with HL, either in univariate or in multivariate analysis. Indeed, most of the previously published studies also failed to show any clear independent prognostic value of various proliferation molecules, with the exception of topoisomerase IIα, in HL (20-25, 29).

In conclusion, the present study for the first time provides data on MCM2 and MCM7 expression in HL; one of the main purposes of our study was to explore and demonstrate the potential effect of expression of these molecules on FFS and OS and our findings suggest that higher MCM7 expression might be an indicator of inferior patient outcome. However, prospective validation in larger number of patients is mandatory before this marker is included in the prognostic armamentarium for patients with HL.

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