Correlation of Minichromosome Maintenance Protein 6 Expression Rate and Clinical Outcome in Patients With Hodgkin's Lymphoma

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Abstract- Minichromosome maintenance complex component 6 (MCM-6) is one of the six proteins of the MCM family, which are involved in the initiation of DNA replication, represents a marker of proliferating cells. The goal of this study was to evaluate the prognostic relevance of the neoplastic cell proliferation rate in patients with Hodgkin's lymphoma (HL). We evaluated the formalin-fixed paraffin-embedded lymph node biopsy specimens from 55 patients by using monoclonal antibody against MCM-6 and compared these findings with clinical data and treatment outcome. Median of MCM-6 expression was 85% (range: 35%-99%). In multivariate analysis, MCM-6 expression, B symptoms, and age were not statistically significant predictor for relapse in contrary to response (P=.001) and stage of disease (P=.048). Patients with lower MCM-6 expression rates showed higher relapse rate and lower disease-free survival (DFS). Meanwhile, patients with MCM-6 expression less than 85% showed shorter DFS (P=.031). We hypothesize that in group of patients with lower MCM-6 expression rate, a larger proportion of proliferating malignant cells are arrested in the very early phase of mitosis, in comparison to the group of patients with higher MCM-6 expression, and this could imply a shorter and probably higher relapse rate in the former group.

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Introduction

Hodgkin lymphoma (HL) is a B-cell-derived malignancy with marked epidemiologic heterogeneity (1). Even though that HL therapy was improved but still some patients experience disease progression. Alterations in genes controlling apoptosis and proliferation of HL cells may be associated with the clinical aggressiveness of the disease (2-4). Different studies have described genetic (1) and socioeconomic factors (for example management of infection notably Epstein-Barr virus) (5) in the development of a subset of patients with HL.

Prognostic factors should help to stratify treatment according to the risk profile and early identification of patients at high risk of recurrence (6). The proliferative activity of tumoral cells has been known as prognostic marker in cancer and may provide important information about treatment failure. For several years a high proliferation rate, measured by counting the number of mitotic figures or of Ki-67 expressing cells (7). The Ki67 antigen presents in the nuclei of cells in all phases of the cell cycle, but it is not expressed in quiescent or resting cells in the G0 phase (8). Considering that Ki67 expression affected by external factors such as nutrient deprivation, the use of this marker for proliferation rate assessment which could lead to underestimation of the number of cycling cells (9). So, the value of prognostic factors has to be updated periodically (10).

Genomic DNA replication occurs only once during the cell cycle. To date, several proteins have been identified that are involved in the initiation of DNA replication, including the origin recognition complex and Minichromosome maintenance complex component (MCM) (11-15). Notably, MCM proteins expression is

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stable throughout the cell cycle, which ideally makes them a much more sensitive marker of proliferation than other proliferation markers which present in all proliferating cells (15). Many studies have focused on the different members of MCM family (16-18). Also, different studies demonstrate that the transition from the cell cycle to quiescence (G0 phase) is due to the down-regulation of the MCM2–7 protein complexes (19).

MCM-6 is one of 6 members of the MCM family, consisting of 821 amino acids with molecular mass of 105 kDa (20). Limited studies have been done on MCM-6, and of those, the most important ones have been done on chondrosarcoma (21) and mantle cell lymphoma (22).

The clinical behavior of HL looks like a low-grade tumor. In the previous study (23), we hypothesized this issue by proliferation arrest of Hodgkin's and Reed-Sternberg cells in the G1 phase of the cell cycle, which is of variable duration and comprises up to 50% of the cell cycle length. Considering that MCM-6 is already expressed in the early G1 phase, it is very useful for determining number of proliferating cells in HL which potentially leads to disease progression.

The aim of this study was to evaluate why MCM-6 expression in Iranian HL patients corresponds to the actual tumor growth and clinical outcome.

Materials and Methods

Biopsy specimens from 55 patients from July 2006 to May 2008 with classic HL diagnosis were reviewed in the department of pathology, National Research Institute of Tuberculosis and Lung Disease (NRITLD), Shahid Beheshti University of Medical Sciences. The diagnosis was established according to the World Health Organization (WHO)/Revised European-American Lymphoma (REAL) classification (24).

The medical records of each patient including the outcome of therapy were reviewed. The staging was done according to Cotswolds modified Ann Arbor staging system for Hodgkin lymphoma (25). The bulky disease is defined as a mass larger than 10 cm (26). The response criteria used in this study were adopted from Report of an International Workshop to standardize response criteria for non-Hodgkin lymphomas by Cheson *et al*., (27).

All patients initially had been treated with standard ABVD regimen (Adriamycin 25 mg/m^2, Bleomycin 10 mg/m^2, Vinblastine 6 mg/m^2 and Dacarbazine 375 mg/m^2 every 2 weeks up to 6 cycles). For bulky or residual disease radiotherapy was performed following chemotherapy. As most patients were alive and exclusion data was very high overall survival was not analyzed. We evaluated the correlation of relapse and Disease free survival (DFS) with MCM-6 expression according to its median cut off points. DFS was defined as the time elapsed between treatment initiation and tumor recurrence or death from any cause, with the exclusion of patients who were lost to follow-up.

Antibodies and immunoperoxidase staining

The sections were stained with monoclonal antibodies against MCM-6 (Kiel, Germany). MCM-6 staining was confined to the cell nucleus. Monoclonal antibody against MCM-6 had been prepared from Department of Hematopathology and lymph node registry, Kiel, Germany (with the permission of Professor R. Parwaresch). For immunohistochemistry 4-5 μm thick sections of paraffin-embedded, formalin-fixed tissue were mounted on 3-amino-propyl- triethoxy-silane pre-treated slides. After deparaffinization and peroxidase pretreatment blocking, antigen retrieval was achieved by boiling the sections in Tris buffer, pH=9 in autoclave (1.1 atmospheres, 121° C for 10 min). Then the slides were incubated for 60 min at room temperature with the primary antibodies: MCM-6 directed against the MCM-6 protein (supernatant, dilution 1:25). Staining was completed with the LSAB2 kit (DAKO, DakoCytomation Company, Denmark) and visualized with diaminobenzidine (28). The previously stained slides for CD30 of these blocks were reviewed for unequivocal identification of neoplastic cells. All sections were stained with antibodies against LCA, CD3, CD20, CD15, and CD30. To evaluate the proliferation rate, the number of MCM-6 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 Hodgkin's and Reed-Sternberg cells with any degree of clear nuclear staining were counted as positive, and the percentage was calculated blindly (Figure 1). The Hodgkin's and Reed-Sternberg cells (RS) cells with any degree of nuclear staining were considered positive, and the percentage was calculated. Reactive lymphocytes in the background also showed nuclear positivity.

Statistical methods

A paired *t*-test was used for comparing the mean MCM-6 expression. The Student's *t*-test was used for testing the equality of means. Univariate and multivariate logistic regression analysis was used for assessing the impact of independent variables on relapse. DFS was

assessed by Kaplan-Meier's curve and log-rank test. All of the statistical tests performed in significant level of α=0.05. SPSS was used for statistical analysis (SPSS Inc, version16, Chicago IL, USA).

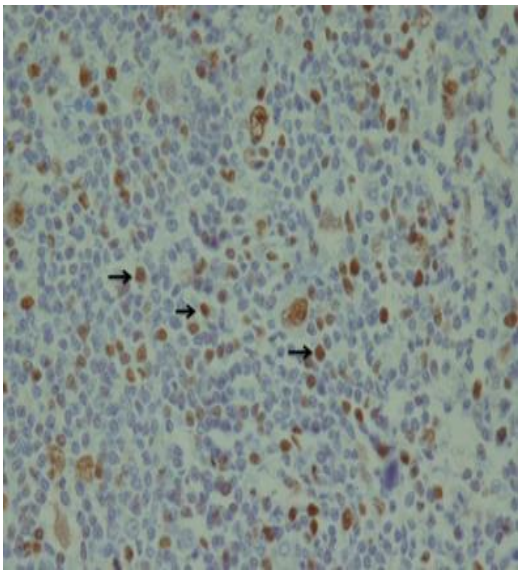

Figure 1. MCM-6 nuclear positivity in reactive lymphocytes (arrows)

Results

This study included 55 patients with a median age of 23 years (range, 13 to 68 years). Clinicopathological characteristics of paints are shown in Table1. Meanwhile, 55.4% of patients also received radiotherapy. At a median follow up of 72 months, 51 of 55 patients (89%) were alive. Eighteen patients (31.6%) had relapse *vs.* 34 (59.6%) who did not (3 cases had missing data for relapse).

Median MCM-6 expression rate was 85% (range: 35-99%). Univariate analysis of MCM-6 expression level revealed only statistically meaningful difference for age (*P*=0.008) (Table 2). Both uni- and multivariate analysis of clinicopathological factors demonstrated that only response to treatment and stage of disease have correlation with increased relative risk for relapse (*P*=0.001 and 0.048, respectively) (Table 3).

Furthermore, we evaluated the correlation of relapse and DFS with MCM-6 expression according to its median cut off points (*i.e.* 85%) (Table 4). Median of DFS for all patients was 73.3 months. Due to large number of censored data in the group of patients with >85% MCM-6 expression, the median duration of DFS have not been reached after 72 months of observation. Median of DFS was 41.4 in the other group. In DFS analysis although the *P* did already show a significant difference (*P*=0.031), indicating that patients with MCM-6 expressions less than 85% have shorter DFS compared to patients with MCM-6 expressions over 85%.

Table 1. Patients' clinicopathological characteristics

Parameter		Number (%)
Age	**Range (yrs)**	13-68
	Median (yrs)	23
Sex	**Female**	33 (60)
	Male	22 (40)
Symptom	**A**	7 (12.8)
	B	48 (87.2)
Stage[a]	**I**	3 (5.5)
	II	22 (40)
	III	17 (30.9)
	IV	13 (23.6)
Subtype[b]	**Lymphocyte rich**	2 (3.6)
	Nodular sclerosis	37 (67.4)
	Mixed cellularity	16 (29)
	Lymphocyte depleted	0 (0)
Bulky disease	**Yes**	28 (50.9)
	No	27 (49.1)

a**:** According to Cotswold's modified Ann Arbor staging system for Hodgkin lymphoma
b: According to WHO/Revised European-American Lymphoma (REAL) classification

Table 2. Correlation between clinical parameters and MCM-6 expression level (univariate analysis)

MCM-6 [a] expression (%)		Mean±SD [b] (%)	Median (%)	Range (%)	*P*
Age (median cutoff)	**≤23**	75.62±17.33	76	35-99	0.008 *
	>23	86.46±10.62	90.5	56-98	
Sex	**Female**	80.55±15.78	86	38-98	0.91
	Male	81.05±15.21	82	35-99	
B symptom	Yes	81.58±15.16	85	35-99	0.27
	No	75±17.37	78.5	50-94	
Stage [c]	**I+II**	83.87±14.79	89	35-97	0.18
	III+IV	78.07±15.82	79.5	38-99	
Response [d]	**CR+CRu+PR**	81.25±14.24	84	35-97	0.53
	Progression	84.86±12.2	87	63-98	
Relapse	**Yes**	75.33±16.98	77	38-98	0.057
	No	83.6±13.17	87.5	35-99	

*Significant *P*

a: Minichromosome Maintenance Protein 6; b: SD: standard deviation c: Cotswolds modified Ann Arbor staging system for Hodgkin lymphoma classification; d: CR complete response ,Cru : The use of the above definition for CR and that below for PR eliminates the category of CRu, PR : partial response.

Table 3. Uni- and multivariate logistic analysis of clinicopathological factors with respect to relapse

Parameter	Reference level	*P* from Univariate analysis	*P* from Multivariate analysis
MCM-6 [a] expression level (median cutoff)	≤ 85% vs >85%	0.122	0.098
Age (years, median cutoff)	≤ 23 vs > 23(Refer to Reviewer comment 4)	0.444	0.731
B-symptom	Yes vs No	0.628	0.809
Stage [b]	I+II *vs.* III+IV	0.026*	0.048*
Response [c]	CR[d] , CRu [e], PR [f] vs Prog [g].	<0.001*	<0.001*

*significant *P* value

a: MCM-6 Minichromosome Maintenance Protein 6, b: Cotswolds modified Ann Arbor staging system for Hodgkin lymphoma; d CR: complete response; e: CRU The use of the above definition for CR and that below for PR eliminates the category of CRu; f: PR partial response; g: prog progressive disease

Table 4. Correlation between cut off point of median expression levels of MCM-6 in relation to relapse and DFS

Cut off point	Relapse		Median of DFS[a]		Mean of DFS[a]		*P*
	Yes n(%)	No n(%)	Estimate ± SD [c]	95%CI[b]	Estimate ± SD [c]	95%CI[b]	
≤85%	12(44.4)	15(55.6)	41.4±18.9 months	4.2-78.6 months	42.6±7.8 months	27.3-57.9 months	0.031*
>85%	6(24)	19(76)	Non-reached [d]	-[d]	67.2±7.3 months	52.8-81.5 months	
Overall	18(34.6)	34(65.4)	73.3±23.1 months	28-118.6 months	54.39±5.7 months	43.2-65.57 months	
Missing data	3(5.4)		--	--	--		

a: DFS disease free survival; b: CI confidence interval; c: SD standard deviation; d: median of DFS was not calculated due to large number of censored data in patients with>85% MCM-6 expression, so, the median duration of DFS has not been reached after 72 months of observation.

*significant *P*

Discussion

To the best of our knowledge, the present study is the first one evaluating the expression of MCM-6 and its potential prognostic significance in Iranian HL patients. MCM-6 expression was observed at a median of 85% of HL cells while the only statistically significant difference was found concerning age. Response to treatment and stage of disease have correlation with increased relative risk for relapse. Inverse correlation of lower MCM-6 expression with increased risk of disease recurrence was seen.

Initially, the relationship between the factors of gender, age, stage, relapse, treatment response and B symptoms and MCM-6 expression rate was evaluated using univariate analysis. We observed that only in the age group under 23-year-old the MCM-6 expression was meaningfully lower, although its clinical importance is

questionable. Notably, the comparison between non-relapsed and relapsed groups showed MCM-6 expression rate was marginally lower in the latter group. Multivariate analysis revealed that only stage and response to treatment affect the relapse rate, which is a rational finding.

Most patients with HL diagnosis have favorable outcome, but some patients may be experienced disease relapse, having no favorable response to standard treatments (29). The reasons for this poorer outcome may link to the specific molecular mechanism influencing the therapy response. Different studies have proposed that increased levels of MCM family may not only be a marker of proliferation (30,31) but may also be sign of precancerous cells and the potential for recurrence (6). Meanwhile, using median expression rate of MCM-6 as cut off point showed that patients with lower expression level have shorter DFS. This inverse correlation of lower MCM expression with adverse prognostic factors is not without precedence in other cancers with other members of MCM family group. Nishihara *et al.*, (32) reported that in colorectal cancer both MCM-2 and MCM-7 expression was lower in Dukes' stage C tumors than in Dukes' stage B ones but, paradoxically, a high index for MCM-7 was shown to be an independent prognostic factor, while that for MCM-2 was not. Also, the proliferation rate of tumor cells did not influence the outcome of HL patients in Tiemann *et al*., survey (19).

We hypothesize that in group of patients with lower MCM-6 expression rate, a larger proportion of proliferating malignant cells are arrested in the very early phase of mitosis, undetected by MCM-6 marker, and make them more resistant to treatment and capable for early relapse (i.e. shorter DFS). On the other hand, the tumors that harbor higher MCM-6 expressions are more sensitive to treatment and consequently more prone to delayed relapse (i.e. higher DFS) and probably lower relapse rate. In conclusion, our results suggest that the accumulation of molecular events seems to be associated with a worse outcome.

Our results propose that specific molecular mechanism may be associated with a worse outcome but recently the interaction pathways of HL cells with non-malignant reactive and stromal cells in lymphoma tissues with different mechanism such as depriving HL cells of important pro-survival signals and immune escape strategies of tumoral cells, (2) was highlighted and must be considered in clinical research. Also, there is no consensus on scoring systems and cut-off values for both proliferation markers. More comprehensive studies with higher number of patients are needed to further validate our findings. Meanwhile, using various proliferation markers with long term follow-up analysis of clinical outcome may be helpful for further evaluation of this subject.

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