Original Article

Is There any Correlation between Ki67 Expression and EBV Infection in Hodgkin Patients in Iran?

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ABSTRACT

Background and Objective: **The association of EBV with Hodgkin Lymphoma (HL) has been intensely investigated over the last few years. EBV is also associated with several other malignancies. On the other hand, Ki67 molecule serves as a widely accepted proliferation marker. Several studies were previously performed about the expression of Ki67 in HD. This study tried to detect the correlation between Ki67 expressions with chronic EBV infection in HD patients of Iran by attenuating confounding factors.**

Materials and Methods: **Hodgkin patients were divided into two groups regarding their EBV infection status. The case and control groups were matched for the stage of the disease. Immunohistochemical methods were used to detect Ki67 expression while DNA extraction and PCR amplification were performed to indicate chronic EBV infection.**

Results: **Clinicopathologic criteria of two groups including male to female ratio, age, presence of B symptoms, and pathologic subtypes were not significantly different. Ki67 expressed in 21% of EBV infected cells while 30% of EBV negative cells had this marker. There was also no statistically significant difference between these two groups.**

Conclusion: **After omitting the possible effect of confounding factors such as the presence of other malignancies and advanced disease stage, there was no correlation between Ki67 expression and EBV infection in Hodgkin patients in this study.**

Key words: Hodgkin's Disease, EBV, Ki67 Expression, Proliferation Cell Index

Introduction

Hodgkin disease (HD) is a lymphoproliferative malignancy which consists one percent of newly diagnosed neoplasms in the United States (1). HD is characterized by disruption of normal lymph node architecture and the presence of large mononucleated (Hodgkin) and multinucleated (Reed-Sternberg) cells among non-neoplastic inflammatory infiltrates (2). Hodgkin lymphoma (HL) is classified into two distinct clinicopathologic entities: nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) representing 5% of all HL cases; has germinal center genotype and is not typically EBV associated (2), and classical Hodgkin lymphoma (CHL) which has a post-germinal genotype. CHL is further divided into four subtypes: nodular sclerosis (NS), lymphocyte rich

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(LR), lymphocyte depletion (LD), and mixed cellularity (MC). Geographic variation significantly affects the distribution of these subtypes (3-5).

Previous studies were contradictory about the relationship between EBV and CHL outcomes (6-10).

Following Thomas Hodgkin's initial description of HL in 1832, there was much controversy as to whether HL was a malignant, inflammatory, or infectious process. The association of EBV with HL has been intensely investigated over the last few years. Despite improvements in our understanding of EBV-associated HL, the true contribution of EBV to the pathogenesis of HL remains unknown (11) but EBV may provide to abnormal B cell survival signals, protecting them from apoptosis.

EBV is however associated with several malignancies. In this regard, 30-50% of HL is EBV-associated (12). On the other hand, Ki67 molecule serves as a widely accepted proliferation marker, which in a number of studies of non-Hodgkin lymphoma (NHL) shows an association with shorter survival (13).

Several studies were previously performed about the expression of Ki67 in HD (14-16). This study tried to detect the correlation between Ki67 expressions with chronic EBV infection in HD patients. It is for the first time that such a subject is studied all over Iran and Middle East. The other positive point of the current study is the omission of the factors that are thought to be associated with the presence of high Ki67 expression such as the high stage of HD and the existence of other malignancies that were not detected at the time of HD diagnosis in our patients.

Materials and Methods

Patients and samples

A total of 217 patients diagnosed with HD were retrospectively collected from four different referral centers in Tehran (see appendix). Those cases that do not fulfill the following criteria were excluded (88 patients); 1. Initial diagnosis was made in lymph node, 2. Parafin-embeded formalin-fix tissue from lymph node was available, and 3. A minimum 2-year follow up was acquired. Meanwhile,

64 cases were omitted because of unsatisfactory staining. Thus, 65 cases were finally eligible for analysis. In addition, 42 HD patients with chronic EBV infection markers were selected as the case group (group I). Furthermore, 23 HD patients with negative EBV terminal protein were designated as control (group II). For finding the pure effect of chronic EBV infection on Ki67 (proliferation cell index) expression, we tried to omit the confounding factors.

Considering the possible effect of advanced disease on cell proliferation, we selected a control group with the same proportion of disease stage. In addition, as occult malignancies other than Hodgkin's disease can alter the Ki67 expression, all the patients were followed for at least 2 years and in case any other malignancy detected, the patient was excluded from the study.

Data are presented as number of cases or means ± standard deviation and are statistically compared for significant difference by Chi-square and two tailed student's t-test. A P value less than 0.05 was considered statistically significant.

Immunohistochemical staining

All procedures on paraffin-embedded sections were performed in a single lab and all the immunohistochemical staining for Ki67 and EBV presence were made in a single center to reduce measurement errors. The sections were cut at 4 micron and sialinized with triethoxylsi lylpropylamin for tissue adherence to slides. Paraffin sections were dewaxed and brought into the water and then Ag retrieval was performed in 0.01 molar citrate buffer in microwave oven at 800 watts for 10 minutes. Slides were cooled down in room temperature, washed quickly in TBS (Tris Buffered Saline, pH 7.4) and stained with anti Ki67 Ab (DAKO A0047 and DAKO LSAB2) kit (K0675) and DAB chromogen (s3000).

The standard immunohistochemical procedure using Streptavidin-biotin-complex labeled alkaline phosphatase was used for preparing the slides before microscopic evaluation.

Quantification

A total area of 3 cores added together (0.84 mm) were examined in every case to count for Hodgkin Reed Sternberg (HRS) cells and the ratio of HRS cells stained with Ki67 antibody to all counted HRS cells and was expressed in percentile. Percentage below 20% was considered negative and the ratio above or equal to 20% was designated positive.

EBV DNA extraction and PCR amplification

Paraffin block slides from lymph nodes of all cases were studied for the presence of EBV by DNA extraction and PCR amplification.

DNA extraction

Slides which contained paraffin block slices were incubated in 65°C water bath for 30 minutes and then

dewaxed in xylene for 15 minutes. In the next step, slides were soaked in 100% and 70% ethanol for 10 and 5 minutes respectively. Slices were removed and transferred to 1.5 ml microtubes. DNA was extracted from paraffin block slices following standard protocols after dewaxing.

PCR amplification

Genomic DNA from each sample was amplified in polymerase chain reaction. Primers for PCR were designed from exon 4, 5 of terminal protein. The sequences of primers are available on request. PCR amplification was carried out in a 25 µl reaction volume containing ~100 ng genomic DNA, 60 ng of each primer, 200 µmol of each dNTP, 1 unit Taq DNA polymerase (Cinagen) in a standard PCR buffer supplied by the manufacturer. Amplification was performed in a Thermocycler (MWG. CO.) under the following conditions: an initial denaturation at 94°C for 2 min was followed by 35 cycles at 94°C for 30 s, 55.5 °C for 30 s, 72 °C for 30 s, and a final extension of 3 min at 72 °C. PCR amplified products were analyzed in a 1% agarose gel for detection of a 182 BP fragment (Figure 1).

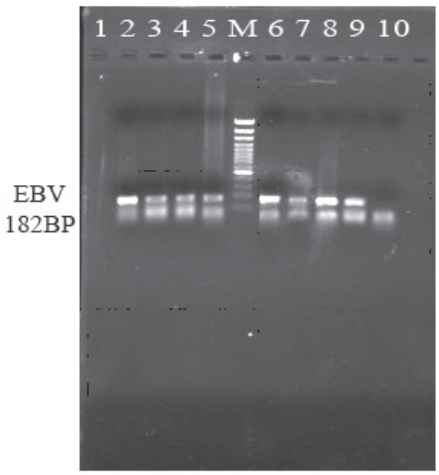

Figure 1. EBV 182 BP fragment in agarose gel

Results

Comparison of EBV-positive and -negative patients Clinicopathologic Characteristics

The results are tabulated in table 1. As a part of matching criteria of the study, there was no significant difference in the proportion of advanced stage HD (stages III and IV) over earlier stages (stages I and II), comparing EBV-infected and EBV-negative patients (respectively 20/22 vs. 11/12; p=0.98).

The male to female ratio was 20/22 in EBV-positive vs. 14/9 in EBV-negative patients. No statistical significant difference was found comparing these ratios (p = 0.3). The average age and presence of B symptoms were nearly the same in these groups.

Table 1. Clinicopathologic comparison of HD patients regarding EBV status in Tehran study

	EBV-positive	EBV-negative	P value
	Group I (n=42)	Group II (n=23)	
Stages			
I and II	22	12	p=0.98
III and IV	20	11	
Sex			
Male	20	14	p=0.30
Female	22	9	
Age	32.86±16.4	29.48±11.15	p=0.07
B symptoms			
Positive	14	8	p =0.90
Negative	28	15	
Pathology			
NS	13	8	#
MC	25	14	
LP	4	1	

Data in this table indicate number of cases except age, which is reported in average year ± standard deviation.

Abbreviations:

NS=*NoduloSclerosis*

MC=*Mixed Cellularity*

LP=*Lymphocyte Predominant*

Ki-67*rs= Ki-67 protein in Reed Sternberg cell*

 # = after combining the cells due to small sample size in each category, P value was more than 0.05.

Expression of Ki67

The results are tabulated in table 2. Comparing the expression of Ki67, there was no statistically significant difference between groups I and II.

Table 2. Comparison of Ki67 (cell proliferation index) expression in HD patients of Tehran study between two groups

	Group I (n=42)	Group II (n=23)	p value
Ki67rs			
Positive	9	7	P=0.42
Negative	33	16	

Discussion

Mixed-cellularity Hodgkin Lymphoma (MCHL) was the most common subtype in our study, which was consistent with similar results of Middle East (18-20). Ki67 protein, a protein detected in G1, S, G2, and M phases of cell cycle but not in G0 is a widely accepted proliferation marker (17). This protein is expressed in a range of 86.7% until 100% in Hodgkin cells (14, 21). It is also expressed in some other malignancies in different rates (13-16). The correlation between Ki67 expression and EBV infection in HD patients has been investigated in several studies, e.g. Kanavarus et al depicted no correlation between the expression of Ki67 and EBV status or histotype of HL (14). This finding was in concordance with Wang et al finding in their study (13). Possible effect of EBV on Ki67 expression was considered in this study because of higher prevalence of EBV and different subtype prevalence (MCHL) in this region as compared to western countries (18-20).

Several factors are thought to affect the higher expression of Ki67 including advance disease stage and the presence of other malignancies (13-16). For reducing the effect of such confounding factors, we matched case and control groups for the proportion of advanced disease stage and followed the patients for at least two years to exclude any other possible malignancies.

This study showed no significant correlation between Ki67 expression and EBV infection even after omitting confounding factors ($p = 0.42$). This result was in concordance with similar studies which was done in western countries (13-14).

Appendix

Patients' data were gathered from the following hospitals in Tehran, Iran:

Naft hospital, Shariati hospital, Imam khomeini hospital, and Arad hospital's Cancer institute

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