Investigation of miR9-1, miR9-2 and miR9-3 Methylation in Hodgkin Lymphoma

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Key Words

Hodgkin lymphoma · microRNA · Methylation

Abstract

Background: miR9 is an important tumor suppressor microRNA regulated by DNA methylation in various types of cancers. **Methods:** We analyzed the methylation status of the 3 members of the miR9 family in 58 cases of Hodgkin lymphoma (HL) in comparison to 15 reactive lymph nodes. We also assessed the relationships between miR9 methylation and Epstein-Barr virus (EBV) infection and several clinicopathological parameters. **Results:** We found that 84.5% of HL cases had a methylation in at least 1 of the 3 loci of miR9, whereas none of the nontumoral samples was methylated. The highest rate of methylation was found in miR9-2 (5q14.3) in 74.1% of the HL cases, followed by miR9-3 (15q26.1) in 56.9% and miR9-1 (1q22) in only 8.6% ($p < 0.001$). The promoter methylation of miR9-3 was more frequent in patients older than 15 years than in children ($p = 0.02$) and among women rather than men ($p = 0.02$). However, no significant correlation was found between miR9 methylation and EBV infection. **Conclusion:** These results indicate that miR9 methylation, especially miR9-2, is a frequent event in HL and may be involved in HL pathogenesis, irrespective of EBV infection.

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Introduction

Hodgkin lymphoma (HL) is a B-cell neoplasm in which the tumor cells represent only 1% of the tumor bulk, with the vast majority of cells being a mixture of infiltrating immune cells and fibroblasts actively attracted via chemokine secretion by the malignant cells, the so-called Hodgkin and Reed-Sternberg cells [1]. HL accounts for about 1% of all cancers and 30% of lymphoid malignancies worldwide [2]. HL is subclassified according to the morphology of malignant cells and the composition of the cellular background into the: nodular-sclerosis, mixed-cellularity, lymphocyte-rich and lymphocyte-depletion subtypes [3].

The Epstein-Barr virus (EBV) is present in the tumor cells in a variable proportion of cases, depending on the geographic region and the ethnicity, age, gender and immunological status of patients [4], but there is no evidence of its role in the onset of disease. It has been reported that several viruses have their own microRNA (miRNA) [5] and that there is an interaction between the host miRNAs and virus miRNAs [6, 7].

miRNAs are short, noncoding RNAs that are thought to regulate gene expression by sequence-specific base pairing in the 3'-untranslated regions (UTR) of the target mRNA, inducing direct mRNA degradation or transla-

tional inhibition [8, 9]. miRNA expression is frequently deregulated in human cancer, and can act as both tumor suppressor and oncogenes [10, 11]. Accumulating studies demonstrate that miRNAs play important roles in angiogenic signaling, cell proliferation, apoptosis avoidance and tumor invasion pathways [12]. Several tumor-suppressor miRNAs are frequently silenced by DNA methylation; one of them is miR9. There are 3 genomic loci that code for the same mature miR9: miR9-1 (1q22), miR9-2 (5q14.3) and miR9-3 (15q26.1) [13]. miR9 has frequently been silenced by hypermethylation in several types of human cancers, including gastric cancer [14], hepatocellular carcinoma [15], breast cancer [16], bladder cancer [17], small-cell lung cancer [18], oral and oropharyngeal squamous-cell carcinoma [19], renal-cell carcinoma [20] and leukemia [13, 21].

Little is known about the role of the hypermethylation of tumor-suppressor miRNAs in lymphomas, and no previous study has investigated the methylation status of miR9 in HL. Our aim was to evaluate the methylation status of the 3 genomic loci encoding miR9 in HL, and to investigate their relationship with EBV infection and several clinicopathological parameters.

Materials and Methods

Patients and Tissue Samples

Samples investigated in this study were clinical cases routinely examined between 2005 and 2012 in the Department of Pathology, Farhat Hached University Hospital, Sousse, Tunisia. Fifty-eight patients were included in the study. Cases were selected on the basis of available paraffin blocks with representative tumor tissue. Patients were eligible for the study if they had no previous treatment or history of malignancy, transplantation or immunodeficiency.

The histological sections of all HL cases were reviewed to verify the presence of tumor cells and evaluate the composition of the reactive cells on the microenvironment (eosinophils, neutrophils, plasma cells, histiocytes and lymphocytes).

The age range of the patients was 5–81 years (median 24 years) and 13 of them (22.4%) were younger than 15 years. The male-to-female ratio was 1.2:1. Nodular sclerosis was the most common histological subtype (67.2%), followed by the mixed-cellularity subtype (22.4%).

The clinical stage at presentation, established according to the Ann-Arbor system, was available for 45 patients; 60% of them were diagnosed as stage III or IV. The majority of patients (58.7%) presented with B symptoms (night sweats, weight loss and/or fever) or as high-risk according to the International Prognostic Score (IPS, 52%). Increased levels of lactate dehydrogenase (LDH) were found in 74.2% of patients.

Patients were uniformly treated at the same institution with standard regimens according to the protocols of the Tunisian Hodgkin Study Group valid at the time of diagnosis. Most of them,

60%, received chemotherapy only, and 40% were treated with chemoradiotherapy. Follow-up data were available for 43 patients. The median follow-up time after diagnosis was 39 months (range 1–98 months). Besides HL samples, we also investigated 15 reactive lymph nodes obtained from patients with nonmalignant diseases (age range 10–62 years, median 43 years; male-to-female ratio 1:1).

DNA Extraction and Methylation-Specific PCR

Genomic DNA was extracted from paraffin-embedded tissues as described previously [22]. Briefly, 3–10 sections (5 µm thick) were homogenized and digested using TE buffer (50 mM Tris HCl at pH 8.5, 1 mM EDTA), 0.5% Tween 20 and proteinase K (10 mg/ml) for 16 h at 56°C, followed by heating at 95°C for 10 min to inactivate the enzyme. Extracted DNA was quantified by a biophotometer (Eppendorf, Hamburg, Germany) and the presence of amplifiable DNA was assessed by amplification of a 268-bp fragment of the human β -globin gene using a set of primers described elsewhere [23].

The methylation status of miR9-1, miR9-2 and miR9-3 was determined by methylation-specific PCR. This assay is based on the selective conversion of cytosine to uracil by sodium bisulfite, followed by PCR amplification using specific primers that allow the discrimination between methylated modified sequences [22].

Genomic DNA from each sample was subjected to bisulfite treatment as previously described [23]. Genomic DNA (1–2 µg) was denatured with 2 M NaOH at 37°C for 30 min (final concentration of 0.2 M NaOH), followed by incubation with 3 M of freshly prepared sodium bisulfite and 10 mM hydroquinone (pH 5.0). The DNA was purified with the Wizard DNA clean-up kit (Promega, Madison, Wis., USA). The DNA modification was completed by the addition of 3 M NaOH for 5 min at room temperature, followed by ethanol precipitation. The pellet was allowed to dry and was then dissolved in 100 µl of distilled water.

To confirm the presence of bisulfite-modified DNA in each sample, amplification of a 133-bp DNA fragment of the β -actin gene was performed, using a primer set [24] which amplifies bisulfite-modified DNA (but not wild-type DNA), irrespective of the methylation status of the sample.

The bisulfite-modified DNA was used as a template for methylation-specific PCR amplifications with primers specific for the methylated promoter of miR9-1, miR9-2 and miR9-3 previously described (100, 110 and 155 bp, respectively) [25]. PCR amplifications were performed in 3 µl of bisulfite-modified DNA templates in 25-µl reaction volumes containing 1 U of Taq DNA polymerase (Promega), 10 Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.25 mM of each dNTP and 0.2 µM of each primer. Amplifications were performed in a Bio-Rad thermal cycler (iCycler, Bio-Rad, Marnes-la-Coquette, France). PCR conditions were as follows: denaturation at 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, for 30 s at the specific annealing temperature and for 30 s at 72°C. The reaction was finished with a 10-min extension at 72°C.

A positive control was included in each experiment; CpG universal methylated DNA (Qbiogene, Carlsbad, Calif., USA) was used as for the methylated alleles. Negative controls without DNA were included in each experiment.

Amplified products were electrophoresed on 2% agarose gels stained with ethidium bromide and photographed under ultraviolet illumination using the Gel Doc 2000 System (Bio-Rad).

Fig. 1. Analysis by methylation-specific PCR of the promoters of miR9-1, miR9-2 and miR9-3 in HL in comparison to reactive lymph nodes. P+ shows a positive control, B represents a negative control (H₂O without DNA template) and lane M shows a 50-pb DNA ladder. Numbers indicate representative examples of methylated (band is present) and unmethylated (band is absent) cases.

EBV in situ Hybridization
In order to identify the EBV-associated cases of HL, in situ hybridization with oligonucleotide probes against EBV-encoded small RNAs (EBERs) (DakoCytomation, Glostrup, Denmark) was performed. Positive staining was recognized as a blue-black color in the nucleus of the neoplastic cells. A known EBV-positive nasopharyngeal carcinoma was used as positive control. All cases with EBER-positive tumor cells were assessed as EBV-positive HL.

Statistical Analysis
Statistical analyses were carried out using SPSS software. Pairwise correlations between the methylation status of miR9 genes and clinicopathological parameters were investigated by the χ^2 test or the Fisher exact test where appropriate. Survival rates were calculated with the Kaplan-Meier method and compared with the log-rank test. Overall survival was defined as the interval from the date of diagnosis to the date of death from any cause or the last follow-up. Event-free survival was defined as the interval from the date of diagnosis to the date of disease progression, relapse, death from any cause or the last follow-up. Independent significance of factors examined was determined using multivariate binary logistic regression analysis based on the proportional hazard model. Probability values of $p \leq 0.05$ were regarded as statistically significant.

Results

Hypermethylation of the Promoters of miR9 Genes

The β -actin gene was used as a methylation-positive control to confirm correct procedural technique. The status of methylation of miR9 genes was determined by methylation-specific PCR for 58 HL patients, comparatively, with 15 nontumoral lymph node samples. Representative results for miR9-1, miR9-2 and miR9-3 methylation-specific PCR are shown in figure 1. We found that 84.5% of HL cases were methylated in at least 1 of the

Table 1. Methylation rates of miR9 genes in HL and cases of reactive lymph nodes

	HL	Reactive lymph nodes
miR9-1	5/58 (8.6%)	0/15
miR9-2	43/58 (74.1%)	0/15
miR9-3	33/58 (56.9%)	0/15
miR9-1/2/3	49/58 (84.5%)	0/15

miR9 genes. Interestingly, none of the nontumoral samples showed hypermethylation in any of the miRs tested.

miR9-2 was methylated in 43/58 HL cases (74.1%) and miR9-3 in 33/58 cases (56.9%), but miR9-1 methylation was detected in only 5/58 cases (8.6%). The differences between the methylation rates of the 3 loci were statistically significant ($p < 0.001$). The three miR9 loci were simultaneously hypermethylated in only 3 (5.2%) cases of HL patients (table 1).

The correlations between miR9 methylation status and the microenvironment elements (eosinophils, neutrophils, plasma cells, histiocytes and lymphocytes) are shown in table 2. No significant correlation was noted.

Correlation between Hypermethylation of miR9 Genes and EBV Infection

EBV infection was investigated by in situ hybridization. Representative examples of the detection of EBV by this approach are presented in figure 2. In situ hybridization analysis showed the presence of EBV in the nuclei of the malignant cells in 19/58 HL cases (32.7%). No significant correlation was found between EBV infection and the methylation of the miR9 genes (table 3).

Table 2. Microenvironment characteristics of HL patients according to miR9 methylation status

	Number of cases	miR9-1			miR9-2			miR9-3			miR9-1/2/3		
		U	M	p value	U	M	p value	U	M	p value	U	M	p value
Eosinophils													
<10%	41	39	2	0.11	10	31	1	15	26	0.11	7	34	0.42
≥10%	15	12	3		4	11		9	6		1	14	
Neutrophils													
<10%	50	45	5	0.55	12	38	0.47	20	30	0.2	7	43	0.62
≥10%	6	6	0		2	4		4	2		1	5	
Plasma cells													
≤15%	36	33	3	0.59	12	24	0.06	16	20	0.74	34	2	0.71
>15%	20	18	2		2	18		8	12		19	1	
Histiocytes													
≤15%	30	27	3	0.56	10	20	0.12	13	17	0.93	29	1	0.44
>15%	26	24	2		4	22		11	15		24	2	
Lymphocytes													
<50%	37	33	4	0.65	8	29	0.3	18	19	0.22	5	32	0.55
≥50%	19	18	1		6	13		6	13		3	16	

M = Methylated; U = unmethylated.

Correlation between Hypermethylation of miR9 Genes and Clinicopathological Characteristics

The analysis of the association between the promoter methylation of the investigated miR9 genes and the clinicopathological parameters was performed on cases with available data; results are shown in table 3. Promoter methylation of miR9-3 was found more frequently in patients aged over 45 years (63.6%) and in adults (67.6%) rather than children (23%) ($p = 0.02$). The promoter methylation of miR9-3 was more frequent among women than men (73.1 vs. 43.7%, $p = 0.02$). These correlations remained significant in the multivariate analysis ($p = 0.03$ and $p = 0.02$, respectively).

The analysis of event-free survival and overall survival was performed on cases with available data. Table 4 presents the survival rates of the univariate analysis of event-free survival and overall survival according to miR9 status. No significant correlation was found between patient survival and miR9 methylation.

Discussion

The promoter hypermethylation of critical-pathway genes, specifically tumor-suppressor genes, is a potential biomarker and therapeutic target for various cancers [26]. Recently, enormous progress has been made in the discovery of microRNAs and in the evaluation of the DNA methylation effect on this new class of small non-

Fig. 2. In situ hybridization for the detection of EBERS in HL. **a** Case showing intense black signal in the nuclei of the neoplastic Hodgkin and Reed-Sternberg cells. **b** An HL case that was EBER-negative. The arrows indicate the tumor cells. $\times 400$.

Table 3. Clinicopathological parameters of HL patients according to the methylation status of the miR9 genes

Parameters	Number of cases	miR9-1			miR9-2			miR9-3			miR9-1/2/3		
		U	M	p value	U	M	p value	U	M	p value	U	M	p value
Age, years													
≤15	13	12	1	0.47	3	10	0.96	10	3	0.02	2	11	0.96
16–45	34	30	4		9	25		11	23		5	29	
>45	11	11	0		3	8		4	7		2	9	
Gender													
Male	32	29	3	0.6	10	22	0.29	18	14	0.02	6	26	0.35
Female	26	24	2		5	21		7	19		3	23	
Histologic subtype													
Lymphocytic-rich	4	4	0	0.71	2	2	0.56	1	3	0.24	1	3	0.51
Nodular sclerosis	39	36	3		9	30		15	24		5	34	
Mixed cellularity	13	11	2		3	10		7	6		2	11	
Lymphocyte depletion	2	2	0		1	1		2	0		1	1	
Ann-Arbor stage													
I/II	18	16	2	0.34	6	12	0.06	7	11	0.29	4	14	0.16
III/IV	27	26	1		3	24		14	13		2	25	
B symptoms													
No	27	26	1	0.36	6	21	0.44	12	15	0.84	5	22	0.19
Yes	19	17	2		3	16		9	10		1	18	
Albumin <4 g/dl													
No	2	2	0	0.91	0	2	0.75	0	2	0.11	0	2	0.75
Yes	21	20	1		3	18		15	6		3	18	
Hemoglobin <10.5 g/dl													
No	21	19	2	0.53	6	15	0.15	11	10	0.51	4	17	0.2
Yes	19	18	1		2	17		8	11		1	18	
WBC count ≥15,000/mm ³													
No	27	25	2	0.7	7	20	0.17	13	14	0.9	5	22	0.12
Yes	13	12	1		1	12		6	7		0	13	
Lymphocyte count <600/mm ³ or <8%													
No	27	25	2	0.66	7	20	0.54	13	14	0.35	5	22	0.34
Yes	6	6	0		1	5		4	2		0	6	
IPS													
>3 (high-risk)	13	13	0	0.48	1	12	0.26	7	6	0.58	1	12	0.46
≤3 (low-risk)	12	11	1		3	9		6	6		2	10	
LDH >250 U/l													
No	8	8	0	0.54	2	6	0.38	5	3	0.17	1	7	0.6
Yes	23	21	2		3	20		8	15		2	21	
Bulky tumor ≥10 cm													
No	35	33	2	0.53	5	30	0.09	15	20	0.27	3	32	0.11
Yes	10	9	1		4	6		6	4		3	7	
Mediastinothoracic ratio, ≥0.35													
No	25	24	1	0.33	3	22	0.25	10	15	0.52	2	23	0.51
Yes	16	14	2		4	12		8	8		2	14	
Extranodal invasion													
No	29	26	3	0.29	6	23	0.62	15	14	0.32	4	25	0.64
Yes	14	14	0		3	11		5	9		2	12	
EBV status													
Positive	19	17	2	0.53	4	15	0.4	9	10	0.42	2	17	0.37
Negative	39	36	3		11	28		16	23		7	32	

Pearson χ^2 or Fisher's exact tests were performed with unknown cases excluded. Numbers in bold type represent significant correlations. M = Methylated; U = unmethylated; WBC = white blood cell.

Table 4. Impact of miR9 methylation on event-free survival and overall survival in HL patients

		Event-free survival, months		Overall survival, months	
		mean ± SD	p value	mean ± SD	p value
miR9-1					
Unmethylated	69.6±6.7	0.16	n.a.	n.a.	
Methylated	26.5±1.5				
miR9-2					
Unmethylated	62.2±9.8	0.46	68.5±8.8	0.87	
Methylated	65.1±7.5				
miR9-3					
Unmethylated	66.6±9.1	0.81	80.1±7.3	0.79	
Methylated	66.4±8.9				
miR9-1/2/3					
Unmethylated	56.3±12.6	0.87	65.3±11.5	0.85	
Methylated	67.1±7.1				
n.a. = Not applicable.					

coding RNAs. The methylation of miR9 genes has been studied in various solid cancers [14–20] and in some hematological malignancies, particularly leukemia [13, 21], but, according to our knowledge, no previous study has reported the methylation status of miR9 loci in HL.

In this study, we tried to clarify whether or not the methylation of miR9-1, miR9-2 and miR9-3 is involved in the formation of a field defect for HL and the relationship with EBV infection and clinicopathological parameters. We found that 84.5% of HL cases had a methylation in at least 1 of the 3 loci, but that none of the nontumoral lymph node samples investigated was methylated. This result suggests that miR9 methylation may be involved in the pathogenesis of HL. miR9 acts as regulator of gene expression by binding with a partial complementarity to the UTR of its targets; it affects cell migration and proliferation in a tumor-type-specific pattern by means of the B-cell differentiation regulators NF- κ B [27] and PRDM1 [28]. Another putative target for miR9 is the UTR of Bcl-6, which has one conserved predicted binding site. Bcl-6 is known to play a role in terminal B-cell [29] and T-cell differentiation [30].

Interestingly, the highest rate of methylation in our study was found in miR9-2 (74.1%) and then miR9-3 (56.9%), with miR9-1 being methylated in only 8.6% of the HL cases. This difference is statistically significant ($p < 0.001$). No significant differences in the methylation rates between miR9 loci have been reported in other studies on nonlymphoid neoplasms [14, 15, 31, 32]. This ob-

servation suggests that miR9-2, located on 5q14.3, is the main locus affected in HL. Previous reports support this observation; indeed, earlier cytogenetic studies showed that the 5q chromosome is frequently altered in various types of hematological malignancies, including leukemia [33, 34], non-HL [35, 36] and HL [37]. It has been shown that the long arm of chromosome 5 harbors a large number of genes coding for hematopoietic growth factors or their receptors [38]. A recent genome-wide association study of 1,200 HL patients reported an association between EBV-negative HL and rs27524 (5q15) and rs20541 (5q31). We found no correlation between miR9 methylation and the EBV infection in HL.

Viruses may participate in the origin of some tumors, such as the EBV in HL. The involvement of the EBV in DNA methylation has been documented in previous works on nasopharyngeal and gastric carcinoma [39–41]. It has been suggested that the interaction between viruses and the malignant cells might be mediated partly by miRNAs. We hypothesized that the EBV infection may induce changes in miR9 methylation in HL, but we found no correlation between miR9 methylation and the EBV infection in the HL cases tested. In support of our finding, Navarro et al. [42] found no correlation between EBV status and miR9 expression in HL. However, they reported that other miRNAs showed a variety of expression patterns, depending the EBV status. In EBV-positive HL cases, miR-96, miR-128a, miR-128b, miR-129 and miR-205 were underexpressed whereas miR-28, miR-130b, miR-132, miR-140 and miR-330 were overexpressed.

The analysis of the relationship between miR9 methylation and the clinicopathological parameters of our patients showed that miR9-3 methylation was more frequent in patients over the age of 15 years than in children ($p = 0.02$). Furthermore, the promoter methylation of miR9-3 was more frequent among women than men (73.1 vs. 43.7%, $p = 0.02$). Few studies have investigated the methylation of miR9 in hematological malignancies. Rodriguez-Otero et al. [13] examined the methylation of miR9 in acute lymphoblastic leukemia, and found that it was more frequent among children than adults.

With regard to the survival analysis, we did not found a correlation with miR9 methylation. The methylation of this miR was an independent prognostic factor for event-free, disease-free and overall survival in acute lymphoblastic lymphoma [13]. Others studies reported a correlation between miR9 methylation and survival of patients in gastric cancer [14] and non-small cell lung cancers [31].

In conclusion, we investigated the methylation status of the 3 members of the miR9 family in 58 cases of HL in comparison to 15 cases of reactive lymph nodes as nontumoral samples. We found high rates of miR9 methylation in the HL cases (84.5%), but none of the nontumoral samples was methylated. The highest rate of methylation in the HL cases was found in miR9-2 (74.1%) followed by miR9-3 (56.9%), but miR9-1 was methylated in only 8.6%. The analysis of the association between the promoter methylation of the investigated miR9 genes and the clinicopathological parameters showed that the promoter methylation of miR9-3 was more frequent in patients older than 15 years than in children ($p = 0.02$), and among

women rather than men ($p = 0.02$). No significant correlation was found between miR9 methylation and EBV infection or the survival of patients. These results indicate that miR9 methylation is frequently present in HL and may be involved in the formation of a field defect in HL, irrespective of EBV infection. Other studies are needed to confirm this result and to assess the usefulness of miR9 methylation in the diagnosis and prognostic of HL.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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