Original Article

mRNA in situ hybridization analysis of MMP-9 in chronic lymphocytic leukemia

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Summary:

Background: Several factors render chronic lymphocytic leukemia (CLL) an interesting subject for study by researchers. These include marked progress in understanding the molecular biology of normal and neoplastic lymphocytes and recent advances in molecular genetics techniques. Among molecular markers, matrix metalloproteinase-9 (MMP-9), have been widely studied.

Objectives: The aim of the study is to evaluate the role of MMP-9 in the pathogenesis of CLL and to assess its prognostic role.

Patients and methods: A retrospective cross-sectional study done on 60 patients with chronic lymphocytic leukemia compared with 20 controls (anemic patients), all recruited at the Medical City Teaching Hospital laboratories from January 2004 to December 2007. The bone marrow biopsy of each patient was re-examined histologically. MRNA-In situ hybridization was performed to detect MMP-9.

Results: The frequency of MMP-9 positivity was 78.3% (47 of 60 cases). A significant inverse correlation was found between increasing MMP-9 scores and lower PCV level. A significantly larger number of patients with high score were associated with advanced modified Rai stage than patients with low score. In addition, there was a significant positive correlation between increasing scores of MMP-9 and advancing clinical stage.

Conclusion: MMP-9 positivity was high and the score was significantly associated with advanced clinical stage of the disease.

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Introduction:

Tumor progression is a complex, multistage process by which a normal cell undergoes genetic changes that result in phenotypic alterations and the acquisition of the ability to spread and colonize distant sites in the body. Although many factors regulate malignant tumor growth and spread, interactions between a tumor and its surrounding microenvironment result in the production of important protein products that are crucial to each step of tumor progression [1, 2]. The matrix metalloproteinases (MMPs) are a family of degradative enzymes with clear links to malignancy. These enzymes are associated with tumor cell invasion of the basement membrane and stroma, blood vessel penetration, and metastasis. They have been implicated in primary and metastatic tumor growth and angiogenesis, and they may even have a role in tumor promotion [1, 3]. The primary role of MMPs in spontaneous and experimental metastasis has been considered to lie in creating a path for tumor cells to colonize host tissues by virtue of their extracellular matrix degrading ability. MMPs could presumably mediate tumor cell extravasation and disruption of basement membrane, facilitating subsequent invasion [1, 4].

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Patients, materials and methods:

Selection of the patients: This is a retrospective cross-sectional study, whereby archival paraffin-embedded tissue blocks along with the clinical and hematological records of sixty patients with CLL were recruited at the Department of Hematology of the Medical City Teaching Laboratories in the period from January 2004 to December 2007. The patients were newly diagnosed and did not receive prior treatment. The bone marrow biopsies were performed at diagnosis. Paraffin-embedded tissue blocks of twenty control individuals (age and sex matched) along with their hematological reports were also collected. All the control bone marrows were negative for infiltrative lesions and were obtained from patients with anemia due to iron or vitamin B_{12} deficiencies. CLL patients were diagnosed and selected according to the criteria of the International Workshop on CLL (IWCLL) [5]. In this study diagnosis was based on persistent absolute lymphocytosis of more than 10,000 mature-appearing lymphocytes/µL in the peripheral blood and bone marrow aspirate smear with lymphocytes ≥ 30% of all nucleated cells.All patients had peripheral blood prolymphocytes of less than 10%.Clinical staging was done according to the modified Rai staging system [6].

In-situ hybridization:

Sections, 5 µm in thickness were made on positively charged slides (Esco) from all the study sample and control groups and subjected for in-situ hybridization procedure to detect mRNAs of MMP-9. Biotinylated cDNA liquid probes for MMP-9 together with the in-situ hybridization/ detection kit were purchased from Maxim biotech, USA. The kit contains a house-keeping gene probe as a positive control. A target positive control tissue was also used which included sections from invasive ductal carcinoma of the breast for MMP-9.The procedure of in-situ hybridization was conducted according to the manufacturing company. It involved deproteinization of fixed tissue sections mounted on slides by proteinase-k enzyme, hybridization of a denatured biotinylated probe to the target sequence and denaturation of the target mRNAs in tissue sections. The hybridized probe was then detected by streptavidin– alkaline phosphatase (streptavidin-AP) conjugate. Upon addition of the substrate solution which is 5-brom-4 chloro-3 indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT), an intense blue signal appeares at the specific site of the hybridized probe.

Evaluation of the in situ hybridization signal:

Hybridization signals (diffuse, granular or focal), were detected either in the nucleus or in the cytoplasm of CLL lymphoid cells of BM biopsies with good preservation of the morphological details. In most patients, mononuclear cells (fibroblasts, macrophages, and polymorphs) also revealed positive signal of MMP-9. These cells were found as few, isolated, or clustered elements, throughout the stroma and were clearly distinguished from CLL cells both on morphologic basis and because they usually displayed a different signaling intensity (in plus or minus) from CLL cells. Care was taken to recognize these cells and omit them from the evaluation. Normal lymphoid cells present in BM biopsies of control group did not show any signal for MMP-9 (the median BM lymphocyte percentage was 11.5% with a range between 5 and 20%). Therefore, CLL was considered positive when at least 1% of lymphoid cells gave positive hybridization signal.Quantification of in situ hybridization signal was evaluated under light microscopy (X100, X400 and X1000), whereas the counting of positive cells was performed at oil immersion (X1000). Counting of positive cells was conducted in 10 different fields taking their mean for each sample [7].ISH was given percentage scores, based on the number of stained cells. Percentage scores were assigned as: score 1 (low) = 1-25%, score 2 (intermediate) = 26-50%, and score 3 (high) = 51-100% [7]. Statistical analysis was performed with the SPSS16 statistical software program (SPSS Inc. Chicago, IL, USA). Associations between categorical variables were assessed via crosstabulation and chi-square. Spearman correlation was used to correlate variables when at least one variable was ordered. Exact tests were used to calculate the p value. In all statistical analyses, a p value < 0.05 was considered significant.

Results:

The overall frequency of MMP-9 positivity in CLL was 78.33% (47 of 60 cases; 33 males & 14 females) with no statistically significant difference (table 1).No significant difference was found between MMP-9-positive and MMP-9-negative patients when they are subdivided according to modified Rai staging system (table 2).The distribution of the different percentages of MMP-9-scores among the CLL patients is shown in table 3 and figure 1. No statistically significant difference was found between MMP-9-positive males and females regarding score.A significant direct inverse correlation was found between increasing MMP-9 scores and lower PCV levels ($P < 0.001$) as shown in figure 2.A significantly larger number of patients, with high score for MMP-9 signal, were associated with advanced clinical stage than patients with low score ($p < 0.001$) (Table 4). At the same time, there was a statistically significant direct positive correlation between increasing scores of MMP-9-positive CLL cells and advancing clinical stage of the disease ($p < 0.001$) (Figure 3).

Table 1: Distribution of MMP-9 signal in CLL patients according to sex

		Sex		
		Male	Female	Total
MMP-9 signal	Positive	33	14	47
P = N.S.	Negative	12	1	13
	Total	45	15	60

Table 2: Distribution of MMP-9 signal according to clinical stage of the disease

		Sex		
		Male	Female	Total
MP-9 score	Low (1-25%)	8	4	12 (25.5%)
	Intermediate (26-50%)	8	7	15 (31.9%)
P = N.S.	High (51-100%)	17	3	20 (42.6%)
	Total	33	14	47 (100%)

Table 3: Distribution of MMP-9 score according to sex.

		Modified Rai stage			
		Low	Inter-mediate	High	Total
MMP-9 signal	Positive	1	17	29	47
P = N.S.	Negative	0	3	10	13
	Total	1	20	39	60

Table 4: Distribution of MMP-9 score according to clinical stage.

		Modified Rai stage			
		Low	Inter-mediate	High	Total
MMP-9 score	Low 1-25%	1	9	2	12
	Inter-mediate (26-50%)	0	7	8	15
P< 0.001	High (51-100%)	0	1	19	20
	Total	1	17	29	47

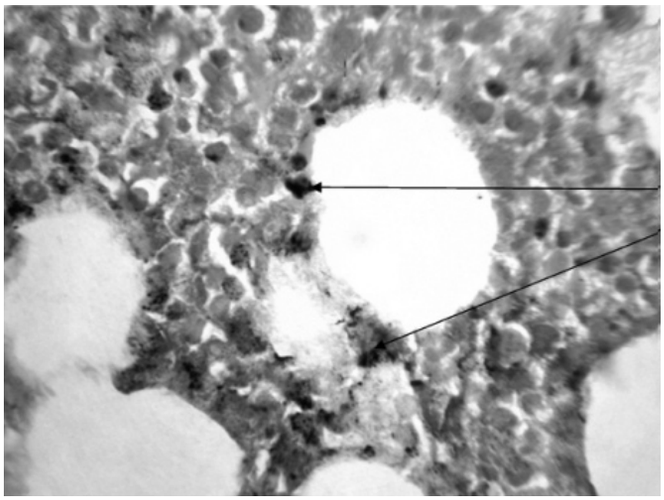

Figure 1. CLL: BM biopsy. Positive MMP-9 ISH signal. The lymphocytes show blue cytoplasmic staining (arrows); intermediate score (× 1000).

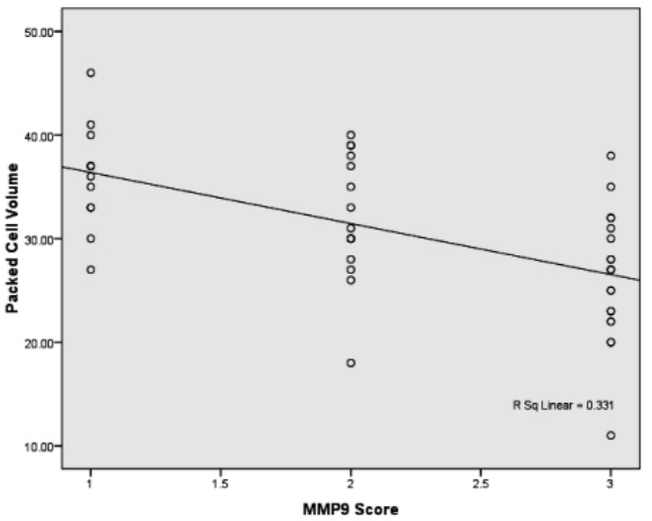

Figure 2. Scatter plot showing correlation between MMP-9 scores and PCV level.
***p=< 0.001**

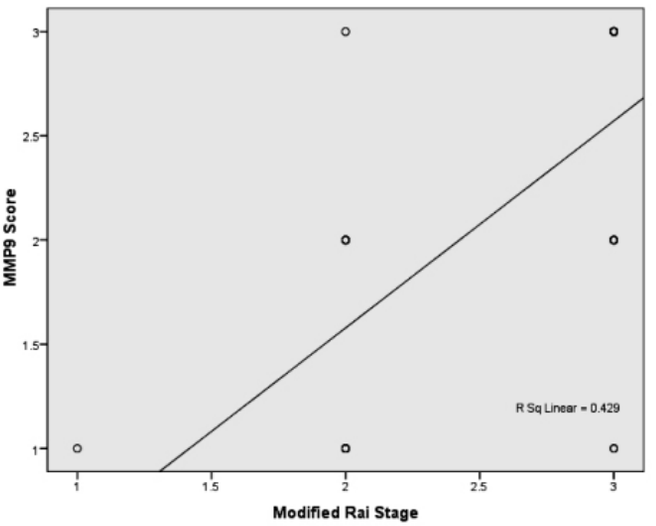

Figure 3. Scatter-plot showing the correlation between MMP-9 score and clinical stage.

Discussion:

We concentrated on MMP-9, because this enzyme is known to be produced by lymphocytes, including normal and certain malignant B-cell types and because they are important for matrix degradation during cell migration and accumulation within tissues [8, 9]. It was therefore important to study MMPs in CLL cells because these enzymes could have a pathogenetic role and consequently be potential therapeutic targetThe frequency of MMP-9 positivity was 78.3% (table 1). Other workers had reported similar results of high frequency of MMP-9 positivity in CLL patients [9, 10]. No association was found between MMP-9 signal positivity and the sex of patients (table 1). Other workers reported similar results [9, 10].

The variable levels of MMP-9 positivity that we observed in CLL cells from different patients are in line with the known heterogeneity of CLL [9].No significant difference was found between MMP-9-positive and MMP-9-negative patients when they are subdivided according to modified Rai staging system (table 2). Other workers reported similar results [9].No statistically significant difference was found between MMP-9-positive males and females regarding score (table 3). Similar results were reported by other workers [9].A significant direct inverse correlation was found between increasing MMP-9 scores and lower PCV levels (figure 2). Low PCV level is an indicators of advanced clinical stage of the disease [11, 12]. This finding indicates that increasing MMP-9 positivity is a stage dependent prognostic indicator. This study indicated that MMP-9 score, as assessed by ISH, is significantly correlated with the clinical stage of the disease (table 4 & figure 3). In the study of Kamiguti et al [9], when they analyzed the levels of MMP-9, in CLL cell lysates, in relation to the clinical stage, they found significant differences between Binet stages A and C, and stages B and C. In their group of patients, high MMP-9 levels were predominantly associated with Binet stage C disease. Thus, the present study is in agreement with the previous study in that increasing MMP-9 positivity is a stage dependent prognostic indicator.

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