

Research Article

Matrix Metalloproteinase-2 Promoter Genotype as a Marker of Cutaneous T-Cell Lymphoma Early Stage

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Received 22 January 2010; Revised 29 April 2010; Accepted 10 May 2010

Academic Editor: Peter J. Oefner

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The aim of the study was to investigate the DNA polymorphic genotype in MMP-2 promoter gene as a potential candidate region for the development of the cutaneous T-cell lymphoma (CTCL) and/or its progression. A total of 89 Czech patients with CTCL (including 23 patients with large plaque parapsoriasis) were compared to 198 controls of similar age and sex distribution, without personal or family history of chronic skin diseases and without personal history of malignancy. The three selected polymorphisms in the promoter of MMP-2 gene (−1575G/A, −1306C/T, and −790T/G) were determined using the PCR-based methodology with RFLP. In our cohort, the associated GGCTT MMP-2 promoter genotype was highly significantly more frequent in CTCL-Ia stage patients compared to patients with parapsoriasis, the tests having high sensitivity and specificity (78%, 83%, resp.). To conclude, use of associated MMP-2 promoter genotype as a DNA marker might make it possible to distinguish between the patients with parapsoriasis and those with CTCL stage Ia, which could substantially improve possibilities of clinical diagnostics, therapy design, and prognosis of this serious condition in the early stages.

1. Introduction

The cutaneous T-cell lymphoma is characterized by skin infiltrating and/or circulating malignant cells displaying a CD4+CD7− phenotype in the majority of cases [1]. CD4+ T lymphocytes from a nonmalignant cloned human T-cell line have been recently reported to produce a 92-kD gelatinase (MMP 9). Apart from production of this 92-kD gelatinase, malignant T-cell clones were reported to produce reduced amounts of a 72 kD gelatinase (MMP 2) [1].

Generally, expression and production of matrix metalloproteinases (MMPs) is associated with advanced-stage tumour disease and MMPs are known to contribute to tumour progression, invasion, and formation of metastases. In patients with mycosis fungoides (MF), the expression of MMP-2 and MMP-9 mRNAs was observed to be significantly upregulated in advanced stages, the expression of either MMP-2 and/or MMP-9 mRNAs consistently rising with advancing disease [2]. V. Apart from their production in MF cells, the MMP-2 and/or MMP-9 mRNAs were found to be

expressed by some stromal cell populations (microvascular endothelial cells, fibroblasts, macrophages), which might be suggestive of the involvement of these cells in the process of tumor invasion. Only weak positivity of the MMP-2 mRNA in microvascular endothelial cells was reported in the control skin samples [2–4], which might be of some importance for further establishment of MMP-2 as a novel clinical marker.

The large repertoire of cells inside the tumour tissue seems to be involved in the process of upregulation of MMP-2 expression level during CTCL progression. Therefore, all the factors influencing MMP-2 gene expression, especially transcription factors, can participate in the process of development of CTCL. The rationale for the study is that variability in the MMP-2 gene promoter can have direct impact on binding of transcription factors, for example, by changing the affinity for given proteins.

MMP-2 gene is located at 16q13. Based on results of comparative genomic hybridization and transcriptomic data, the 16p region was identified as a target region for CTCL-associated gene aberrations [5].

There is a general consensus that MMP-mediated proteolysis is essential for cancer progression and that certain MMPs represent important targets for intervention, effective, and selective inhibition, however, pharmacological intervention in terms of these MMPs remains a major challenge in drug development [6, 7]. Interindividual variability in the MMP genes might also play an important part in investigation of pharmacogenetic aspects of the MMP-modulated therapy.

The aim of the study was to associate DNA polymorphic genotypes in MMP-2 promoter gene with general risk for CTCL development and/or its severity (stages).

2. Material and Methods

2.1. Subjects. A total of 89 patients with CTCL (including 23 patients with large plaque parapsoriasis), diagnosed and treated at the 1st Department of Dermatology of St Ann's Faculty Hospital Brno (55 men and 34 women, median age 62 y, range 26–101 years) were compared to 198 controls (133 men and 65 women, median age 58 y, age range 26–80) without personal or family history of chronic skin diseases and without personal history of malignancy.

The patients with CTCL were classified according to Tumor Node Metastasis (TNM) Classification for Cutaneous T-Cell Lymphoma according to Kashani-Sabet et al. 2001 [8].

In CTCL men, 50% were treated by topical steroids (20% in combination with phototherapy, 2% with retinoids). The CTCL women were treated by topical steroids in 55% (23% in combination with phototherapy, 5% with retinoids). Phototherapy was indicated in 51% of CTCL men and in 51% of CTCL women.

This study was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine, Masaryk University, Brno (no. 64/93, 1993) and was performed in adherence to the Declaration of Helsinki Guidelines. Participants gave their written informed consent which has been archived.

2.2. Genotyping. Genomic DNA was isolated from peripheral leukocytes by a standard technique using proteinase K.

Three MMP-2 promoter polymorphisms (–1575G/A, –1306C/T and –790T/G) were identified by PCR method and restriction analysis according to standard protocols.

Polymorphism –1575G/A MMP-2 was detected by PCR using sense 5'-ACT GAC TCT GGA AAG TCA GAG CA-3' and antisense 5'-GGC ACA GGG TGA GGG GAT GG-3' primers with a PCR product of 269 bp. After restriction analysis with Tsp45I (↓GT G/C AC, New England Biolabs Inc., USA) at 65°C for 4 h, three genotypes could be distinguished in UV light at 2% agarose Serva with ethidium bromide: GG (113 and 156 bp), GA (113, 156 and 269 bp), and AA (269 bp).

Polymorphism –1306C/T MMP-2 was detected by PCR using sense 5'-CTT CCT AGG CTG GTC CTT ACT GA-3' and antisense 5'-CTG AGA CCT GAA GAG CTA AAG-AGC T-3' primers with a PCR product of 193 bp. After restriction analysis with XspI (C↓TAG, Takara, Japan) at

37°C for 4 h, three genotypes could be distinguished: CC (188 bp), CT (188, 162 and 26 bp), and TT (162 and 26 bp).

Polymorphism –790T/G MMP-2 was detected by PCR using sense 5'-GGG TCT TTG TGA CCT CGA TC-3' and antisense 5'-GGT AAA ATG AGG CTG AGA CCT G-3' primers with a PCR product of 118 bp. After restriction analysis with PvuI (CGAT/CG, MBI Fermentas, Lithuania) at 37°C for 3 h, three genotypes could be observed in UV light at 3% Metaphor agarose with ethidium bromide: TT (118 bp), GT (118 and 19 + 99 bp), and GG (19 + 99 bp) [9].

2.3. Statistical Analyses. In all investigated cohorts, distributions of genotype and allelic frequencies and their differences were calculated using χ^2 tests. Consistency of genotype frequencies with the Hardy-Weinberg equilibrium was tested using χ^2 test on a contingency table of observed versus predicted genotype frequencies.

Odds ratio (OR) and 95% confidence interval were calculated to estimate the risks related to detected polymorphisms. To calculate the significance of OR, Fisher's exact test was used. Sensitivity and specificity of results were calculated by Clinical Calculator 1 (<http://faculty.vassar.edu/lowry/clin1.html>). The Holm's test was employed for appropriate correction to multiple comparisons. The program package Statistica v. 8.0 (Statsoft Inc., Tulsa, OK) was used.

3. Results

3.1. Case-Control Study. No significant differences in case-control analysis, neither in genotype distributions or allelic frequencies of all investigated MMP-2 polymorphisms were observed, when performing the analyses separately for the individual polymorphisms (Table 1). Similarly, no significant differences were observed between men and women, neither in the CTCL nor control cohort (data not shown).

All the three polymorphisms were found to be in linkage disequilibrium. When investigating the frequency of associated genotype (GGCCTT), that is, the most frequent in the general population, no differences between patients with CTCL and control subjects were observed (37 patients with GGCCTT/52 patients with other genotype versus 100 control subjects with GGCCTT/98 control subjects with other genotype, $P = .101$). Similarly, no differences in the frequencies of GGCCTT genotype were observed between men and women, neither in the CTCL cohort nor the controls.

3.2. Case-Case Study. However, highly significant differences were observed when comparing the frequencies of the GGCCTT associated MMP-2 genotypes between the specific stages of CTCL. Based on our results, the GGCCTT MMP-2 promoter genotype is significantly more frequent in CTCL-Ia stage patients compared to all other CTCL groups including parapsoriasis (Table 2). All comparisons retained their significance after appropriate correction for multiple comparisons (P_{corr}). The GGCCTT genotype was observed to be the most sensitive and specific marker to distinguish between CTCL patients with stage IA and the patients

TABLE 1: Three promoter MMP-2 gene polymorphisms: patients with CTCL versus control subjects.

				-1575/G			
Genotypes and alleles	GG (%)	GA (%)	AA (%)	P_g	G	A	P_a
CTCL ($N = 89$)	48 (54)	37 (42)	4 (4)	0.931	0.747	0.253	0.804
Controls ($N = 198$)	105 (53)	82 (41)	11 (6)		0.737	0.263	
				-1306 C/T			
Genotypes and alleles	CC (%)	CT (%)	TT (%)		C	T	
CTCL ($N = 89$)	46 (52)	41 (46)	2 (2)	0.492	0.747	0.253	0.853
Controls ($N = 198$)	105 (53)	83 (42)	10 (5)		0.740	0.260	
				-790 T/G			
Genotypes and alleles	TT (%)	TG (%)	GG (%)		T	G	
CTCL ($N = 89$)	46 (52)	39 (44)	4 (4)	0.091	0.736	0.264	0.258
Controls ($N = 198$)	100 (51)	73 (37)	25 (12)		0.689	0.311	

P_g = P -value of a difference in genotype distribution

P_a = P -value of a difference in allelic frequencies.

TABLE 2: Risk for GGCCTT associated genotype in MMP-2 gene promoter in different CTCL stages compared to CTCL-Ia.

	MMP-2-GGCCTT (%)	MMP-2-the others (%)	Odds ratio**	P -value (P_{corr} ***)
LPP* ($N = 23$)	4 (17)	19 (83)	0.07 (0.01–0.28)	.00004 (0.0002)
CTCL-Ia ($N = 23$)	18 (77)	5 (23)		
CTCL-Ib ($N = 24$)	10 (42)	14 (58)	0.10 (0.06–0.71)	.01 (0.02)
CTCL II ($N = 11$)	3 (25)	8 (75)	0.10 (0.02–0.55)	.007 (0.03)
CTCL III+IV ($N = 8$)	2 (30)	6 (70)	0.09 (0.01–0.61)	.01 (0.02)
Controls ($N = 198$)	100 (51)	98 (49)	0.28 (0.10–0.79)	.009 (0.03)

*LPP- large plaque parapsoriasis

**Odds ratio for GGCCTT in any group compared to CTCL-Ia (95% confidential interval)

*** P_{corr} = P -value corrected for multiple comparison.

with parapsoriasis (sensitivity is 78%, 95% CI = 56–92%; specificity is 83%, 95% CI = 60–94%).

When evaluating the risk of GGCCTT MMP-2 genotype for development of parapsoriasis, we observed higher frequencies of the GGCCTT genotype in all other CTCL groups as well as in control subjects (Table 3). In this case, the differences were significant when comparing the CTCL-Ia patients and the control subjects.

4. Discussion

Early diagnosis of CTCL is an extremely important point for efficient decision-making in the treatment of CTCL. However, there is no generally accepted consensus on the range of criteria that should be employed to achieve as early diagnosis of the disease as possible [10]. The differential diagnosis between early stages of mycosis fungoides and large plaque parapsoriasis (both considered a form of CTCL) can be often difficult, especially when being based merely on evaluation of clinical and histological parameters [11]. Therefore, there is a strong need for novel markers that would make the earlier diagnosis of the disease possible; for example, CD13 (aminopeptidase N) was recently identified as an early prognostic factor in CTCL [12]. Based on our results, the variability of the MMP-2 promoter region

might act as potential biomarker of different CTCL stages, especially as for the CTCL stage IA and large plaque parapsoriasis. This is to the best of our knowledge the first study of given SNPs in MMP-2 gene on CTCL cases, which was carried out on a highly static population of the South Moravia, of a Central-European Caucasian origin. Not surprisingly, it will be necessary to replicate the results of the study on larger population samples of different ethnicity to achieve a final conclusion of the importance and significance of the MMP-2 gene variability in CTCL.

Taking into account the structure of DNA in the given locus, the GGCCTT genotype in the promoter of MMP-2 gene should have a high transcription rate. Two functional SNPs *in vitro* in the MMP-2 promoter were recently investigated: –1575 G to A, which alters binding of oestrogen receptor [13], and –1306 C to T, which alters binding of Sp-1. The C, but not T allele of –1306 C/T MMP-2 polymorphism contains an Sp1 motif (CCAACC box), therefore displaying strikingly higher promoter activity *in vitro* [14]. The transcription factor Sp1 is a DNA-binding protein which interacts with a variety of gene promoters containing GC-box elements. The factor regulates the expression of a number of genes participating in multiple aspects of tumorigenesis, such as angiogenesis, cell growth, and apoptosis resistance. The Sp1 levels in untransformed

TABLE 3: Risk for GGCCTT associated genotype in MMP-2 gene promoter in different CTCL stages compared to parapsoriasis.

	MMP-2-GGCCTT (%)	MMP-2-the others (%)	Odds ratio*	P-value (P_{corr}^{**})
LPP* (N = 23)	4 (17)	19 (83)		
CTCL-Ia (N = 23)	18 (77)	5 (23)	17.10 (3.95–73.95)	.00004 (0.0002)
CTCL-Ib (N = 24)	10 (42)	14 (58)	3.39 (0.88–13.08)	.07
CTCL II (N = 11)	3 (25)	8 (75)	1.78 (0.32–9.85)	.400
CTCL III+IV (N = 8)	2 (30)	6 (70)	1.58 (0.23–10.90)	.497
Controls (N = 198)	100 (51)	98 (49)	4.85 (1.59–14.76)	.002 (0.008)

*LPP - large plaque parapsoriasis

**Odds ratio for GGCCTT in any group compared to LPP (95% confidential interval)

*** P_{corr} =P-value corrected for multiple comparison.

cells must be tightly regulated as Sp1 overexpression leads to the induction of apoptosis. Recently, Sp1 has been shown to be overexpressed in a number of human cancers, whereas its overexpression is supposed to contribute to malignant transformation [15].

Furthermore, *in silico*, three transcription factors could be found in the DNA sequence including the T allele of -790 G/T MMP-2 polymorphism: KLF4 (Kruppel-like factor 4, S8 and Evi1-ectopic viral integration site 1 encoded factor (according to the Transfac Matrix Table (MatInspector V2.2)). KLF4 represents the essential transcription factor which is critical and selective for establishment of the proper barrier function of the skin [16]. KLF4 was proved to interact with beta-catenin and to inhibit Wnt signalling [17]. MMP-2 seems to be a Wnt target gene because two TCF sites typical for this gene were identified in MMP-2 gene promoter, interestingly in a close vicinity of the -790 G/T polymorphism (at -861 and -804 positions). Wnt signalling was observed not only to induce expression of matrix metalloproteinase 2, but also to regulate transmigration of T cells [18]. Wnts probably induces a T-cell phenotype capable of migration through collagen [18]. This ability is crucial for extravasation of T cells, which is not observed in parapsoriasis as a rule. Finally, this could explain the observed difference in variability of promoter of MMP-2 gene between the patients with parapsoriasis and CTCL-Ia stage. Last but not least, Evi1 is a highly conserved transcription factor that was shown to upregulate cell proliferation, to impair cell differentiation and also to induce malignant transformation [19].

To conclude, the frequency of the GGCCTT MMP-2 genotype was the lowest in the group of patients with parapsoriasis, when compared to the control subjects. On the other hand, the efficient form of MMP-2 promoter seems to be associated with CTCL Ia stage. We propose an idea that the detection of MMP-2 promoter polymorphism might contribute to resolution between specific stages in the framework of CTCL cohort, that is, between the patients with parapsoriasis and CTCL Ia stage, whereas the test has satisfactory sensitivity and specificity. From the clinical point of view, the GGCCTT MMP-2 genotype can be considered a potential DNA marker that might be employed to assist in clinical resolution between CTCL Ia and parapsoriasis. Our study continues in newly diagnosed patients with large plaque parapsoriasis and with all stages of CTCL, examination of MMP-2 gene expression will be performed

from bioptic skin lesion samples by RT-PCR method. This enables to evaluate the actual functional association between the promoter MMP-2 genotype and MMP-2 expression in the skin of patients with a different stage of CTCL. Definitely, further studies on larger population sample are necessary that could substantially complete possibilities of clinical diagnostics, individualize therapy design, and improve prognosis of the serious disease with respect to variability of MMP-2 gene.

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