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Key Words

Human endogenous retroviruses · Cutaneous T-cell lymphomas · Mycosis fungoides · Reverse transcription polymerase chain reaction · Sézary syndrome

Abstract

Background: Mycosis fungoides (MF) and Sézary syndrome (SS) are the most frequent cutaneous T-cell lymphomas (CTCL). Human endogenous retroviruses (HERVs) were reverse transcribed and integrated into primate chromosomal DNA, becoming noninfectious, although various stimuli may reactivate them. HERV expression seems to be impaired in several human diseases but limited data regarding CTCL are available. Objective: To evaluate the endogenous retroviral transcription profile in CTCL and their expression among disease clinical stages. Methods: Peripheral blood mononuclear cells from 42 MF/SS patients were analyzed. Total RNA was extracted and amplified with reverse transcription polymerase chain reaction. Results were compared with those obtained in a cohort of 20 healthy donors.

Results: HERVs were significantly overexpressed in MF/SS patients compared with healthy donors. No differences were found between early and advanced CTCL stages.

Conclusion: HERVs can act as promoters in MF/SS pathogenesis. It remains to link HERV hyperexpression to the outcome in CTCL patients.

Introduction

Mycosis fungoides (MF) is characterized by longstanding, scaly patch lesions preferentially involving the buttocks and body areas infrequently exposed to sunlight and by a slow evolution over years from patches to plaques and eventually tumors or erythroderma. Lymph node and visceral involvement, as well as large cell transformation, usually occur in the late stages of the disease [1] . Sézary syndrome (SS) is an erythrodermic cutaneous T-cell lymphoma (CTCL) with leukemic involvement, an aggressive clinical behavior and poor prognosis [2, 3] .

The etiopathogenesis of MF and SS remains obscure. Persistent antigen stimulation could lead to a continuous proliferation of T cells and chronic inflammation and, ultimately, to the development of a malignant T-cell clone [4] . Another hypothesis suggests that specific viral agents may serve as triggering

factors [5] . Different viruses have been proposed to have a role in the etiopathogenesis of CTCL, mainly the human T-cell leukemia virus and the Epstein-Barr virus [6, 7] . Conflicting results have emerged from studies investigating the role of the Epstein-Barr virus in CTCL [8] . Our group demonstrated in other reports that the Epstein-Barr virus, parvovirus variants (B19, LaL1/K71, V9) and HHV-7 were not involved in the etiopathogenesis of CTCL [9, 10] . The human endogenous retroviruses (HERVs) are composed of many families of endogenous retroviruses that were integrated into the germ DNA of the primate line over the last ~ 30 million years [11] . After the integration process, during the evolution of the germ line, a series of postinsertional mutations, deletions and recombinations occurred and made the totality of them on infectious in humans, even if replication-competent endogenous retroviruses have been found in other mammals [12] . Nowadays, HERVs are classified in 50–200 families depending on the primer binding site sequences complementary to the 3 ′ end of a cellular tRNA used for reverse transcription [13, 14] . The possibility of HERVs to modify cell gene expression has been emphasized. Solitary long terminal repeats (LTRs) were generated from existing HERVs by the loss of internal sequences upon recombination between the 5 ′ and 3 ′ LTR sequences within the same copies or between separate LTRs [15] . The transcription of HERV-K and other HERV elements is usually suppressed by epigenetic factors such as DNA methylation and heterochromatin silencing by histone modifications. Inserted LTRs act as alternative promoters to stimulate the expression of nearby genes, causing the activation of oncogenes or the inactivation of tumor suppressor genes [16] . Moreover, HERV expression seems impaired in several diseases, ranging from autoimmune to neoplastic disorders [12, 17] . However, various stimuli such as chemical, radiation or other exogenous viral infections may reactivate HERV transcription [18] . Even if the role of HERV-H in the pathogenesis of leukemias and lymphomas has been recently postulated [19] , to date limited data are available regarding the expression of HERV families among CTCL. Recently, Maliniemi et al. [20] detected a significantly increased HERV-W transcription level on MF lesional skin. In a previous study Contreras-Galindo et al. [21] demonstrated HERV-K upregulation in lymphomas. More recently, Fischer et al. [22] described this feature also in chronic lymphocytic leukemia. The purpose of this study was to evaluate the overall endogenous retroviral transcription profile in blood samples from a large cohort of MF/SS patients.

Subjects and Methods

Patients and Samples

The present study was performed in compliance with the principles of good clinical practice and according to the principles of the Declaration of Helsinki. All the patients were included after providing their written informed consent, and the study was approved by the Ethics Committee of the 'A.O.U. Città della Salute e della Scienza di Torino'. Peripheral blood mononuclear cells were obtained from 42 CTCL patients (23 MF and 19 SS). The diagnosis of CTCL was made according to the standard clinical and immunopathological findings [1, 23] . Among the 19 patients with peripheral blood involvement (stage IVA1, evaluated on whole blood by means of flow cytometry using a FACSCanto II cytometer; Becton Dickinson, San José, Calif., USA), the amount of circulating atypical cells ranged from 18 to 98% of total lymphocytes (median: 69%). All SS patients showed an identical clone both in skin and blood. MF/SS patients were classified according to the new TNM staging of the Mycosis Fungoides Cooperative Group 3. Results were compared with those obtained in a cohort of 20 healthy donors of the same geographical area matched for sex and age.

Reverse Transcription

Total RNA was extracted using the TRI Reagent kit (Sigma) according to the manufacturer's protocol. In detail, 1 µg of total RNA was reverse transcribed with 5 µl of polymerase chain reaction (PCR) buffer II 10×, 11 µl of MgCl 2 25 m M , 2 µl reverse transcriptase MuLV 50 U/l (murine Moloney leukemia virus), 1 µl of

RNase inhibitor 20 U/l, 5 µl random hexamers 50 µ M (Applied Biosystems, Foster City, Calif., USA), 1 µl mix dNTPs 100 m M (Amersham), and distilled deionized water in a final volume of 50 µl. The reaction mix was carried out in a GeneAmp PCR system 9700 Thermal Cycle (Applied Biosystems) under the following conditions: 10 min at 20 ° C, 45 min at 42 ° C and 5 min at 99 ° C for the inactivation of enzyme; the cDNAs were stored at − 80 ° C. Relative Quantification by Real-Time PCR Relative quantification of mRNA expression of selected genes was achieved by means of TaqMan amplification and normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was chosen as a reference gene, using the ABI PRISM 7300 Sequence Detection System (PE Applied Biosystems). The expression of HERV-K and HERV-W polymerases and GAPDH was quantified by real-time PCR. Approximately 100 ng cDNA were amplified in a 20-µl total volume reaction containing 2.5 U ampli-Taq Gold DNA polymerase (Applied Biosystems), 1.25 mM MgCl 2 and 900 nmol of the following specific primers: HERV-K primers KPOLF-5 ' -CACTGTAGAGCCTCCTAAACCC-3 ' [24] , KPOLR-5 ' -TTGGTAGCGGCCACTGATTT-3 ' and probe KPOLP-6FAM-CCCACACCGGTTTTTCTGTTTTCCAAGTTAA- TAMRA or HERV-W primers WPOLF-5 ' -ACMTGGAYKRTYTTRCCCCAA-3 ' , WPOLR-5 ' -GTAAATCATCCACMTAYYGAAGGAYMA- 3 ' and probe WPOLP-6FAMTYGGGATAGCCCYCATCTRTTTGGYCAGGCA- TAMRA or GAPDH primers GAPDHF-5 ' -CAAGGTCATCCATGACAAC- 3 ' , GAP DHR-5 ' -GTGGCAGTGATGGCATGGAC-3 ' and probe GAPDH-6FAM-TGGTATCGTGGAAGGA-3 ' GB. KPOLR-5 ' -TTGGTAGCGGCCACTGATTT-3 ' and probe KPOLP-FAMCCCACACCGGTTTTTCTGTTTTCCAAGTTAA- TAMRA were designed with software primer express version 3.0 (Applied Biosystems). The amplifications were performed on the ABI 7500 real-time PCR system (Life Technologies, Austin, Tex., USA) in a 96-well plate at 95 ° C for 10 min followed by 40 cycles of 95 ° C for 15 s and 60 ° C for 1 min. Each sample was run in triplicate. Furthermore, in order to confirm that there was no DNA genomic contamination, control PCR was performed with RNA before reverse transcription using the same primers and probes described above (fig. 1). Relative quantification of target gene expression in patients compared with normal samples was performed with the ΔCt method and the relative Toll-like receptors; hence, results are expressed in corresponding arbitrary units (AU).

Statistical Analysis

Statistical analyses were performed using the two-tailed Student t test and one-way or two-way ANOVA test using Prism software (GraphPad Software, La Jolla, Calif., USA). In all analyses, $p < 0.05$ was taken to indicate statistical significance. Results The study group enrolled 42 CTCL patients of MF/SS type (28 males and 14 females) who were diagnosed, treated and followed up at the Dermatology Clinic of the University of Turin. CTCL patients presented with different clinical stages, including early-phase (9 stage IA, 4 stage IB, 5 stage IIA) and advanced-phase disease (5 stage IIB and 19 stage IVA1). The median age was 70 years (range 41–101). As expected, patients with advancedphase disease were older than those with early-phase (72.5 vs. 67 years old). All patients were receiving an active treatment depending on their stage (topical steroids and phototherapy for early stages, bexarotene or interferon for stage IIA/IIB, extracorporeal photopheresis with or without bexarotene for stage IVA1); 2 stage IIB and 6 stage IVA1 patients were receiving chemotherapy at the time of study entry (pegylated doxorubicin or gemcitabine). Both HERV-K and HERV-W were significantly overexpressed in MF/SS patients compared with healthy donors ($p = 0.0015$ and $p = 0.0001$, respectively) (fig. 2). No differences in the overall amount of HERV-K and HERV-W were found. Interestingly, no differences were found in HERV-K and HERV-W levels comparing early (IA–IIA) and advanced (IIB–IVA1) CTCL stages ($p > 0.05$) (fig. 3) or according to the presence of blood involvement. Moreover, in patients with peripheral blood involvement, no relationships were found between the number of circulating Sézary cells and HERV expression levels. Similarly, no differences were found in HERV-K and HERV-W levels according to age, gender and type of treatment (skin directed versus systemic).

Discussion

The purpose of this study was to evaluate the overall endogenous retroviral transcription profile in blood samples from a representative cohort of MF/SS patients at different clinical stages. Our findings show that MF/SS patients are characterized by the presence in the blood of an overexpression of both HERV-K and HERV-W sequences. This increase does not depend on the stage of disease, demographical features or treatment approaches. The only other paper published in the literature exploring the role of HERV in the pathogenesis of CTCL analyzed a total of 34 samples comprising MF and psoriasis skin lesions, showing HERV-W significantly increased transcription in MF lesions compared with intact skin from the same patient [20] . HERVs represent a large group of endogenous retroviruses (up to 200 families) that were integrated into the germ cell chromosomal DNA of primates, losing their capacity to be infective [11] . The reactivation of HERV transcription consequent to various stimuli such as chemical, radiation or other exogenous viral infections could be implicated in the development of several malignancies through the modification in cell gene expression. Even if our data come from the analyses of blood samples only, they appear in agreement with those of Maliniemi et al. [20] . Together these findings may strengthen the hypothesis that HERVs play a role as promoters in the pathogenesis of MF/SS. Otherwise, it must be taken into account that chronic antigen stimulation could also trigger HERV hyperexpression as a consequence of cytokine production and inflammation [25, 26] . It remains also to ascertain whether the high HERV values we found in CTCL patients are somehow related to the clinical outcome. Actually, no differences in HERV expression levels according to clinical stage and number of circulating Sézary cells were found. Moreover, the lack of difference in HERV-K and HERV-W expression in this group of patients could support the hypothesis that these HERV families act with a similar mechanism. Similarly, the potential role played by treatments in HERV yperexpression also remains to be clarified. In our experience, patients treated by systemic chemotherapy showed a similar HERV expression to those treated by skin-directed treatments, even if the small number in our series limits the statistical power of these findings. No data exist in the literature as to CTCL; a recent paper in breast cancer patients suggested that chemotherapy could lower HERV expression [27] , whilst the development of HERV-K (HML-2) antibodies was reported to have an important value as an indicator of chemotherapy success in patients with germ cell tumors [28] . The existence of a skin-specific HERV transcription profile in different inflammatory dermatoses is a suggestive scenario supported by a previous finding of HERV expression in psoriatic skin [29] . It should be interesting to evaluate the presence of HERV amplification in both the lesional skin and blood of the same patient to ascertain the possible existence of a CTCL-specific HERV transcription profile.

Disclosure Statement

The authors report no conflicts of interest.

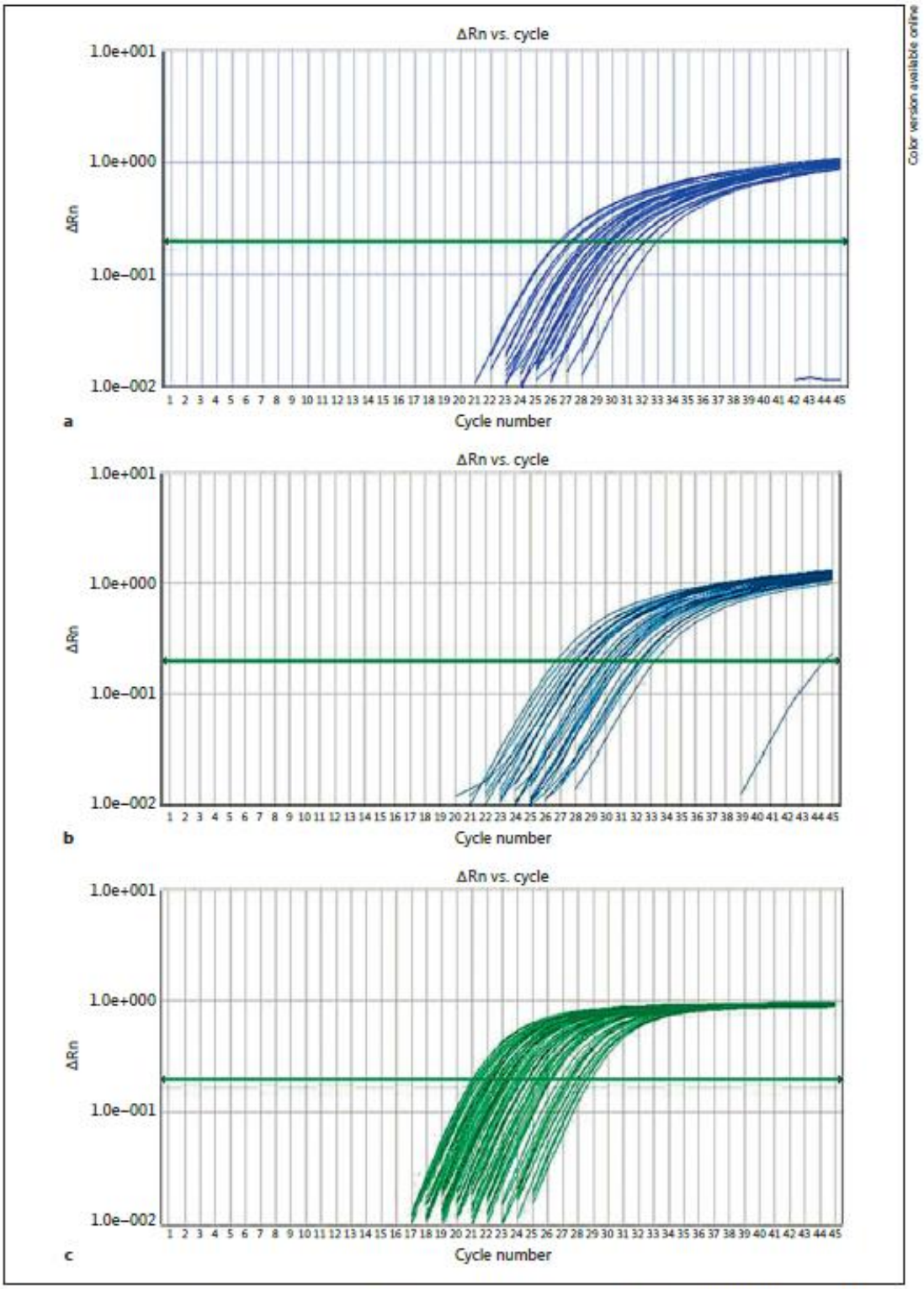

Fig. 1. Example of amplification pattern of HERV-K polymerase-specific cDNA (**a**), HERV-W (**b**) and housekeeping gene GAPDH (**c**).

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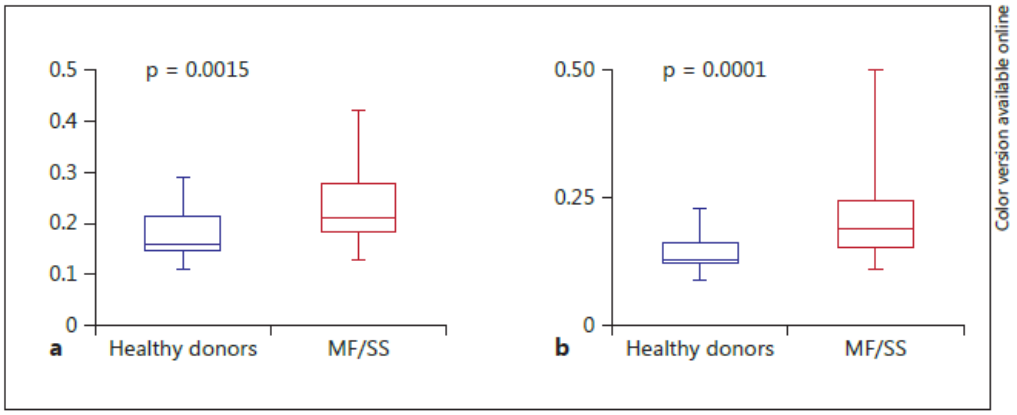
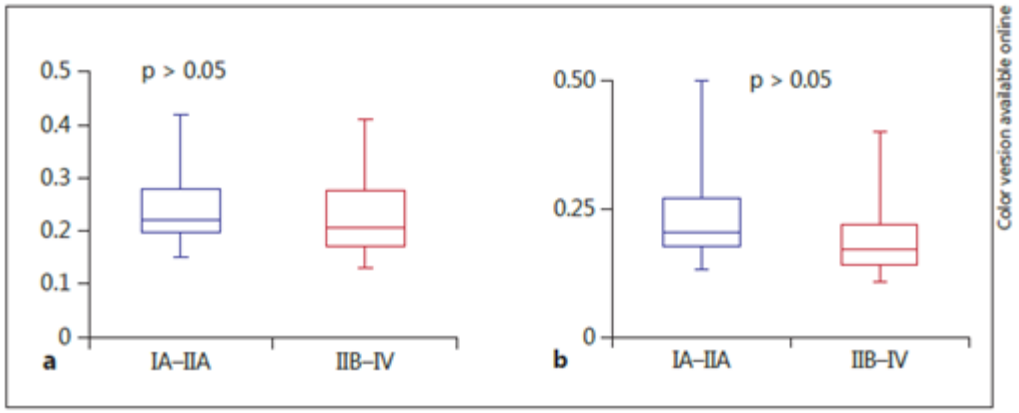


Fig. 2. HERV-K (a) and HERV-W (b) expression (ΔCt method) in healthy donors vs. CTCL (MF/SS).



F ig. 3. HERV-K (a) and HERV-W (b) expression values among stages.

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