

Mycosis fungoides in European Russia: No Antibodies to Human T Cell Leukemia Virus Type I Structural Proteins, but Virus-Like Sequences in Blood and Saliva

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Key Words

Mycosis fungoides · European Russia · Human T cell leukemia virus · *tax* sequences · Antibodies

Abstract

Objective: Mycosis fungoides (MF) is the most frequent form of cutaneous T cell lymphoma (CTCL). Human T cell leukemia virus type 1 (HTLV-1) involvement in MF progression is a matter of debate. The goal of the investigation was to search for HTLV-1 markers in a group of MF patients from a nonendemic area to HTLV-1. **Materials and Methods:** Fifty MF patients and 60 healthy donors from Moscow and the Moscow region were examined for HTLV-1 markers by Western blot, PCR, nested PCR, PCR/Southern hybridization, TaqMan real-time PCR and sequencing. **Results:** Plasma samples from MF patients were repeatedly negative for antibodies to HTLV-1 structural proteins. HTLV-1 *tax*-related sequences (corresponding to the second exon) were found in blood from 20 of 50 MF patients and in 3 of 5 saliva specimens. Three of 8 sequenced *tax*-like amplicons were identical and 5 of 8 contained 1–2 substitutions. *tax* transcripts and antibodies to p40^{tax} were detected in some 'PCR-*tax*'-positive MF patients. Defective HTLV-1 genomes were demonstrated in 2 of 50 MF patients. Phylogenetic analysis

of the defective genome 5'-LTR sequence revealed a relationship with HTLV-1a sequences from the transcontinental subgroup of HTLV-1. **Conclusions:** HTLV-1 *tax*-like sequences were revealed in blood and for the first time in saliva from MF patients living in an HTLV-1 nonendemic region. Expression of *tax*-like sequences was confirmed by both reverse transcription PCR and Western blot.

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Introduction

Human T lymphotropic virus type 1 (HTLV-1) is the causative agent of adult T cell leukemia/lymphoma (ATL) [1, 2] and a chronic progressive demyelinating disease known as tropical spastic paraparesis (TSP) or HTLV-1-associated myelopathy (HAM) [3–5]. Additionally, HTLV-1 association to some autoimmune diseases including uveitis [6] has been shown.

HTLV-1 is transmitted through horizontal and vertical routes. The major routes of horizontal transmission are blood transfusion and sexual contacts, and the vertical route is breast feeding [5]. Besides blood, seminal fluid and milk, saliva might contribute to virus horizontal transmission [7–9].

The HTLV-1 genome is stable, and no specific disease-associated genetic markers were found in patients with different HTLV-1-associated pathologies. In general, HTLV-1-infected individuals have antibodies to structural proteins of the virus. However, it has been reported that some virus carriers and individuals with HTLV-1-associated diseases do not have detectable antibodies to HTLV-1 structural proteins [10–14]. Most virus carriers remain asymptomatic during their life, and the cofactors required for disease progression are largely unknown.

Nucleotide analyses of HTLV-1 isolates of different geographic origins revealed three major subgroups of the virus: Cosmopolitan, Central African and Melanesian [15, 16]. A more recent classification based on gp21 and LTR phylogenetic analysis divided HTLV-1 genotypes in four major subtypes: (A) Cosmopolitan (including Western, Central, Northern and Southern African, Caribbean, Japan and India isolates), (B) Central African, (C) Australo-Melanesian and (D) New Central African [17].

Besides complete HTLV-1 proviruses, defective genomes (dgs) were detected in ATL, TSP/HAM patients, asymptomatic carriers and in cell lines derived from patients' lymphocytes [14, 18, 19]. Most of the dgs contained large internal deletions and are replication incompetent [20]; however, some of them appear to be active and might code for different chimeric proteins [19]. Any contribution of dgs to a particular disease progression is obscure.

Cutaneous T cell lymphoma (CTCL) is a heterogeneous group of malignant T cell lymphomas with primary manifestations in the skin. Mycosis fungoides (MF) is the most common form of CTCL and is a slowly progressing lymphoproliferative malignancy with skin infiltrated by neoplastic T lymphocytes. The disease can spread to lymph nodes or to other organs, such as the spleen, lungs or liver. When large numbers of tumor cells are found in the blood, the condition is called the Sézary syndrome [21].

Patients with MF have varying risks for disease progression or death. The most important clinical predictive factors for survival include patient age, T classification and the presence of extracutaneous disease. Frequently, at the early stages of MF, some other cutaneous diseases may be mimicked, which can sometimes make MF early diagnostics problematic.

The etiology of MF remains unknown and HTLV-1 involvement in MF is still a matter of controversy. Most MF patients are HTLV-1 seronegative in diagnostic tests, and thus, fail to pass WHO serological criteria for HTLV-1 infection [22].

The HTLV-1 *tax* oncoprotein plays a crucial role in virus-associated pathogenesis [23], but Tax is not included as an antigen in diagnostic serological tests. HTLV-1 *tax*-only sequences and antibodies against HTLV-1 Tax were described in MF patients from the United States [24–28]. Other groups revealed HTLV-1 dgs in peripheral blood mononuclear cells (PBMCs) and cutaneous lesions of MF patients [29, 30]. However, several groups failed to detect *tax*-like sequences in MF patients from the United States [31, 32].

Transmission of virus-like sequences without a complete virus is difficult to explain. Some of the results suggest that *tax* transmission occurs with mononuclear cells [27], where *tax* may be present as an extra chromosomal sequence [33].

European Russia (Moscow and the Moscow region in particular) is considered to be an HTLV-1 nonendemic region, and ATL and TSP/HAM are extremely rare [34]. However, MF is not less common than reported in HTLV-1-endemic regions. Thus, it was of special interest to analyze *tax* prevalence among Russian MF patients.

We analyzed the prevalence and expression of *tax* and other HTLV-1 sequences among 50 MF patients from European Russia. All patients were HTLV-1 seronegative. *tax* sequences were detected in the blood of 20 out of 50 MF patients and in saliva of 3 out of 5 patients. This is the first report of *tax*-like sequences in saliva from MF patients. It may suggest an additional way of horizontal transmission of *tax*-like sequences. Expression of *tax* was demonstrated by reverse transcription (RT)-PCR and Western blot.

Material and Methods

Patients and Donors

Blood specimens from 50 (stage IIA–IVA) MF patients (29 men and 21 women, mean age 62 years) diagnosed clinically and dermatologically and 60 blood donors (25–55 years old, mean age 38 years) from Moscow or the Moscow region were studied. All patients and donors were of Caucasian origin. Blood specimens from MF patients were collected at the Department of Dermatology, M.F. Vladimirovsky Moscow Region Clinical Institute. Blood specimens from healthy donors were obtained from the Department of Hematology at the N.N. Blokhin Cancer Research Center RAMS.

Blood and Saliva Processing

PBMCs were separated on a Ficoll-Hypaque gradient, washed twice in phosphate-buffered saline (PBS) and stored at -70°C until use.

Four to five milliliters of whole unstimulated saliva were collected in sterile plastic tubes from 7 MF patients and 6 healthy individuals (laboratory personnel). Saliva specimens (1 ml) were di-

luted 1:3 in ice-cold TN buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA) with protease inhibitor cocktail Complete (Roche Diagnostics GmbH, Mannheim, Germany) and centrifuged at 2,000 *g* for 10 min at 4°C. Pellets were resuspended in 200 µl of TN buffer and used for DNA extraction.

DNA and RNA Extraction

We have taken all possible measures to avoid crosscontamination, including work in separate rooms equipped with especially designed 'DNA extraction' and 'PCR boxes', special sets of pipettes and solution for DNA/RNA extraction being kept in aliquots for the individual specimen processing. Total genomic DNA was extracted from 300 µl whole blood or 5 × 10⁶ lymphocytes in 200 µl PBS or 200 µl saliva pellet (from 1 ml of saliva) using a QIAamp DNA Mini Kit or QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The amounts of genomic DNA isolated from saliva varied from 10 to 40 ng/µl. The time interval between saliva donation and DNA extraction greatly influences the quality and yield of DNA obtained. Total RNA was isolated from 3 × 10⁶ PBMCs using RNazol B (Biogenetics, Poole, UK). RNA extractions from pellets were carried out with a NucleoSpin RNA virus kit (Macherey-Nagel, Düren, Germany). All procedures were performed according to the manufacturer's protocol. Concentration of DNA and RNA was measured using a DU 640B spectrophotometer (Beckman, USA).

Isolation of Viruses

A standard differential centrifugation procedure of virus isolation from plasma was used. Fresh plasma specimens (5 ml each) from 3 'PCR-*tax*'-positive MF patients were centrifuged at 1,500 *g* for 5 min, followed by centrifugation of the supernatant at 10,000 *g* for 10 min. Supernatants were collected and centrifuged at 110,000 *g* for 90 min. Pellets were dissolved in 100 µl of TN buffer (10 mM Tris-HCl, pH 7.6, 15 mM NaCl), laid onto a 20% sucrose cushion and centrifuged at 110,000 *g* for 90 min. All manipulations with the supernatant and pellets were carried out on ice and centrifugation was performed at 4°C. Pellets were dissolved in 20 µl of TN and used for DNA and RNA extraction. Virus particles from the supernatant of the MT-2 cells were isolated and purified as described [35]. Virus preparations were used as antigen for Western blot analysis.

Serum Analysis

Serum samples (dilution 1:100) were examined by particle agglutination assay HTLV-1/2 kit (Murex, USA) and by Western blotting using virus (preparation is described below) isolated from MT-2 cells as an antigen and a commercial Western blot kit (HTLV-1 Blot 2.4, Genelabs Diagnostics Pte Ltd., Singapore). In accordance with published guide lines [22], samples were considered positive when immunoreactivity was observed with at least two Gag and one Env protein. Monoclonal antibodies to p40^{tax} and recombinant protein (rp40^{tax}) were kindly provided by Dr. Françoise Bex (Université Libre de Bruxelles, Belgium).

Protein Electrophoresis and Western Blot

Protein electrophoresis was performed in 10–20% gradient polyacrylamide gels (Novex, Invitrogen, USA). Rainbow markers (Amersham, UK) were used for gel calibration. After electrophoresis, proteins were blotted on a nitrocellulose membrane (BA83, Schleicher & Schuell, Dassell, Germany) for 2 h at +4°C. Mem-

branes were blocked with 7% dry milk (Merck, Germany) in PBS with 0.1% Tween 20 overnight at +4°C. After 2 h of incubation with a corresponding serum (dilution 1:100) and five washes (5 min each) in PBS Tween 20, membranes were exposed to secondary antibodies, namely antihuman IgG-AP (Roche Diagnostics GmbH) in dilution 1:2,000. After five washes in PBS Tween 20, color was developed with BM-purple substrate (Roche Diagnostics GmbH).

PCR and RT-PCR

Primer sets for PCR/nested PCR were synthesized by TIB MOLBIOL (Berlin, Germany) and DNA-Technology (Moscow, Russia).

The list of the primer sequences and their genome location (Reference J0 2029) are shown below (5' to 3'):

5'-LTR – GAC AAT GAC CAT GAG CCC CAA
(nucleotides 24–44);

gag f – GAT ACG AGC GCC CCT
(nucleotides 795–809);

5' to ATG – CCC TTT ATT CCC TAG GCA
(nucleotides 806–823);

A1 – CAA ATC TTT TCC CGT AGC GCT AGC
(nucleotides 824–847);

A2 – TCC AGT TAC GAT TTC CAC CAG TTA AA
(nucleotides 938–963);

gag r – TGG AAA TCG TAA CTG GAG G
(nucleotides 954–936);

SK110 – CCC TAC AAT CCA ACC AGC TAG
(nucleotides 4758–4776);

SK111 – GTG GTG GAT TTG CCA TCG GGT TTT
(nucleotides 4942–4919);

B1 – GGA GGC GAT GTA GTT GCA ATA
(nucleotides 6704–6684);

B2 – GTG TGC TTG GTT TAC AGG GAT
(nucleotides 6678–6660);

tax 6798 f (long) – TCC AAC TGT CTA GTA TAG CCA T
(nucleotides 6798–6819);

tax 7351 f (short) – AGG GTT TGG ACA GAG TCT
(nucleotides 7334–7351);

tax 7537 r (short) – GGA TAA GGA ACT GTA GAG C
(nucleotides 7537–7519);

tax 7571 r (long) – TAA GGA CCT TGA GGG TCT TAG
(nucleotides 7591–7571);

SK43 – TTT CGG ATA CCC AGT CTA CG
(nucleotides 7356–7375);

SK44 – GAT AAC GCG TCC ATC GAT GG
(nucleotides 7512–7493).

Diagnostic *pol* (SK110-SK111) and *tax* (SK43-SK44) primers were as published [36].

For each PCR reaction, 500 ng to 1 µg DNA (equivalent to approximately 75,000–150,000 cells) was used. Hot start PCR amplification was performed as proposed by the supplier of Taq polymerase (Perkin-Elmer/Roche, Germany). Amplifications were carried out in PE 2400 and PE 9700 (Perkin-Elmer) cyclers. After the initial denaturation step at 95°C for 5 min, DNA was subjected to 35–42 cycles of amplification, denaturation at 95°C for 30 s, annealing from 48 to 60°C (dependent on primers) for 40 s, extension at 72°C for 30 s to 1 min. After the last cycle, extension was carried out at 72°C for 10 min, and a 10-µl aliquot of each PCR mixture was run on a 1.8% agarose gel and visualized by ethidium bromide staining. Purity and equal amounts of DNA in specimens were

confirmed by β -actin-specific TaqMan PCR. High-fidelity PCR was performed with the 5'-LTR-*pX* pair primers and amplification conditions suggested by the kit supplier (Roche). Long PCR fragments were analyzed on 1% agarose gel with ethidium bromide.

RNA specimens were treated with DNase 1 (Roche Diagnostics GmbH) at 37°C for 30 min, followed by heat inactivation of the enzyme. RT-PCR was performed using a Titan one-tube RT-PCR kit according to the protocol of the supplier (Roche Diagnostics GmbH). RT reaction was carried out for 1 h at 48°C followed by 43 cycles of PCR in a PE 9700 (Perkin-Elmer, USA) cyclor.

Real-Time PCR

Real-time PCR (quantitative PCR) was done as published [37]. The reaction mixture consisted of 25 μ l of Universal Master Mix (Perkin-Elmer, Germany) containing dNUTPs, MgCl₂, reaction buffer and AmpliTaq Gold; 250 nM of each primer (SK43-SK44) and 100 nM of fluorescence-labeled HTLV-1 *tax*-specific TaqMan probe. DNA and water were added to a final volume of 50 μ l. A 5- and a 50-copy standard of *tax*-containing plasmid (preparation in cooperation with GenExpress, Berlin, Germany) were used as controls. Real-time PCR was performed in Applied Biosystems SDS 7700 or SDS 7000 as follows: 5 min at 50°C, 7 min at 94°C, 45 cycles for 30 s at 94°C and 1 min at 55°C. After TaqMan PCR, the results were analyzed with the Sequence Detector Software (Applied Biosystems, Weiterstadt, Germany).

Southern Transfer and Hybridization

After electrophoresis in a 2% agarose gel, amplimers were transferred overnight in 20 \times SSC on a Hybond N membrane (Amersham). Southern hybridization with digoxigenin-labeled probes was done using a High Prime DNA labeling and detection kit (Roche Diagnostics GmbH). Prehybridization, hybridization, washing conditions and reaction development were as described in the manufacturer's protocol. pMT-2-42 clone containing defective HTLV-1 provirus (gift from Prof. M. Hatanaka, Institute of Virus Research, Kyoto, Japan) and PCR-amplified *tax* fragment (part of the second exon) were used as probes. Gel calibrations were done with digoxigenin-labeled DNA molecular weight markers III and VI (Roche Diagnostics GmbH).

Sequence Analysis

PCR amplimers were purified on 2% low-melting point agarose (BRL, Gaithersburg, Md., USA) and sequenced directly using an ABI Prism dye terminator cycle sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems/Perkin-Elmer, USA) and Applied Biosystems automatic DNA sequencer (Model 377). Sequence analysis was carried out using Lasergene analysis software (DNASTAR Inc.).

Phylogenetic Analysis

Phylogenetic analysis was performed on a 374-bp-long 5'-LTR fragment (part of dg) detected in a patient with MF (Accession No. AF515451). Nucleotide sequences were aligned using the computer software Clustal W [38] with minor manual modifications. Pairwise genetic distances were estimated by Kimura's 2-parameter method [39]; phylogenetic trees were then constructed by the neighbor-joining method [40]. The reliability of the neighbor-joining and parsimony trees was evaluated by analyzing 1,000 bootstrap replicates [41]. The sequence of the Kas-MF1 patient was aligned with all available HTLV-1 LTR prototypic strains for the other subtypes.

HTLV-1c isolate Mel5 was defined as an outgroup. All DNA sequences used for construction of the phylogenetic tree have been previously published [15–17, 42–46].

Results

Plasma Analysis

Plasma specimens from 50 MF patients from Moscow and the Moscow region were analyzed by HTLV-1 particle agglutination assay, followed by Western blot using commercial and laboratory-made diagnostic membranes. All serum specimens (taken in dilution 1:100) were negative for antibodies to HTLV-1 structural proteins. To confirm the data, second blood samples from the same MF patients were taken 4 weeks later and reexamined by Western blot, as described above. All samples were again found to be negative, indicating a stable absence of antiviral immune response.

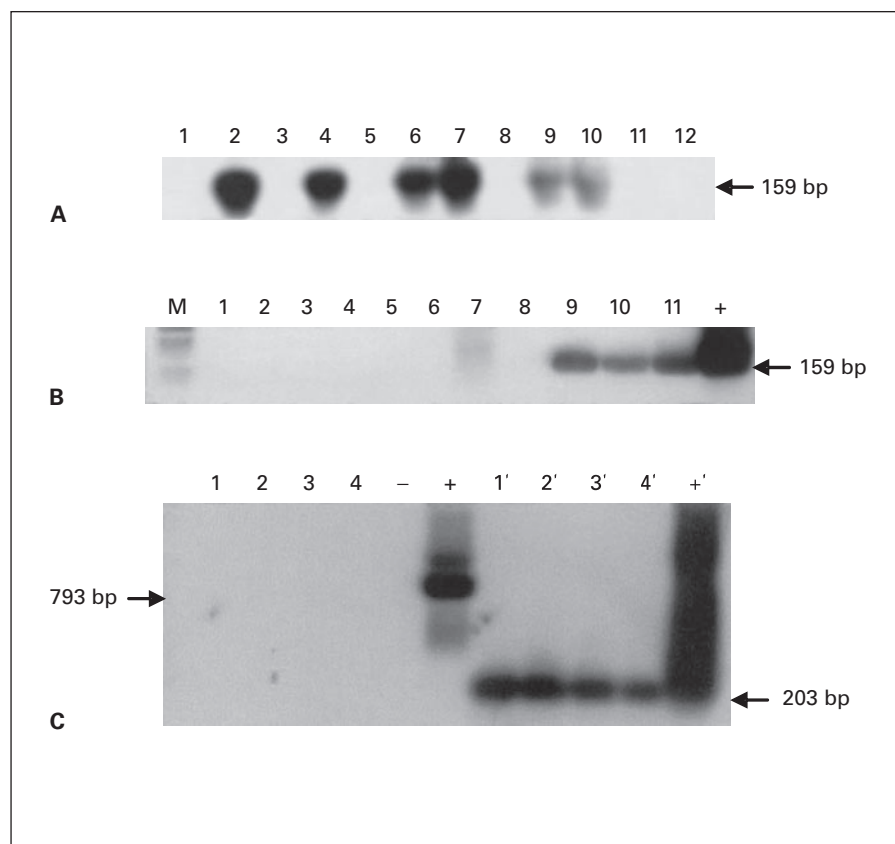
HTLV-1-Like Sequences in Blood and Saliva

DNA specimens from blood of healthy donors and from blood and saliva of MF patients were used for PCR analysis. Initial analysis of DNA samples was carried out by PCR with *tax* second exon primers (SK43-SK44). Twenty out of 50 DNA specimens from MF patients were found positive after PCR/Southern hybridization. The results obtained on 10 specimens from MF patients are shown in figure 1A. None of 60 blood donors, representing our control group, were positive when tested by PCR with diagnostic HTLV-1 *tax* primers followed by Southern hybridization. All DNA specimens were negative for HTLV-1 *gag* sequences by PCR/Southern hybridization.

Saliva might play a role in HTLV-1 transmission since saliva from HTLV-1 carriers contains infected cells and virus particles [7–9]. A search for the *tax* sequences in saliva from MF patients was never done before. To examine whether *tax*-like sequences are present in saliva of MF patients, we isolated genomic DNA from saliva of 5 'PCR-*tax*'-positive MF patients and examined it by PCR followed by Southern hybridization. Three of 5 specimens were positive (fig. 1B), indicating the possible role of saliva in transmission of *tax*-like sequences.

To estimate the size of the *tax*-related fragments in MF patients, two sets of primers were designed that were to 3' and to 5' to the SK43 and SK44 target sequences, respectively. Four DNA specimens (isolated from blood) that were initially PCR 159 bp '*tax* positive' were tested with these primers. A 203-bp-long fragment was detected

Fig. 1. Detection of HTLV-1 *tax*-like sequences in blood and saliva specimens by PCR/Southern hybridization. **A** HTLV-1 *tax*-like sequences in 5 of 10 blood specimens from MF patients. Line 1: negative control (H₂O), line 2: positive control (DNA from MT-2 cells), lines 3–10: DNA specimens from MF patients. Amplimers (159 bp long) are indicated. **B** HTLV-1 *tax*-like sequences in 3 of 5 saliva specimens from MF patients (blood ‘PCR-*tax*’ positive). M = Digoxigenin markers VI. Lines 1–5: saliva specimens from healthy donors; line 6: negative control (H₂O); lines 7–11: saliva specimens from MF patients; line +: positive control (DNA from MT-2 cells). Amplimers (159 bp long) are indicated. **C** Rough estimation of the *tax*-like sequence length in MF patients by PCR with two sets of primers. Lines 1–4: DNA from 4 ‘PCR-*tax*’-positive (SK43-SK44) MF patients amplified with a ‘*tax* long’ (6798 and 7591) pair of primers (793-bp-long amplimer); lines 1’–4’: DNA from 4 ‘PCR-*tax*’-positive’ (SK43-SK44) MF patients amplified with a ‘*tax* short’ (7334 and 7537) pair of primers (203-bp-long amplimer); lines + and +’: positive controls (DNA from MT-2 cells), respectively; line -: negative control (H₂O). Positions of the amplimers are indicated.



by PCR/Southern hybridization in all 4 DNA specimens using the *tax* ‘short’ pair of primers. However, no positive signals were detected after PCR with the *tax* ‘long’ pair of primers covering a 793-bp-long fragment of the *tax* region (fig. 1B), indicating that *tax*-like sequences in MF patients are between 200 and 790 bp in length.

Since *tax*-like sequences were present in low copy numbers (most were detected only after PCR/Southern hybridization), utilization of inverse PCR (that usually requires at least 300 copies) to justify possible integration was not feasible.

In blood DNA specimens from MF patients by long-range PCR with 5’-*gag*-SK44 pair of primers, we failed to detect HTLV-1-like sequences of ‘quasi’ genomic size. By PCR with 5’-LTR-*pX* (B1 and B2)-specific primers using standard PCR conditions, we detected a defective HTLV-1 genome in two ‘PCR-*tax*’-positive MF patients. Both amplimers were nearly 200 bp shorter than the amplimer revealed in MT-2 cells (data not shown). One of the amplimers was purified and sequenced. Seven unique substitutions were revealed in the 374-bp-long LTR fragment when compared with HTLV-1 ATK sequence.

Sequence Analysis

Purification of the amplimers shorter than 150 nucleotides is a technical problem mostly because of primer dimers that ‘contaminate’ the amplimer and the proximity of amplimer size to the cutoff of the purification columns. We obtained good purification of 8 amplimers that were sequenced in both directions.

It has been well documented that HTLV-1 *tax* sequences are conserved. Sequence analysis of purified amplimers revealed that 3 of 8 were 100% identical and indistinguishable from numerous sequences available in GenBank. However, 5 of 8 differed in 1–2 nucleotides. Nucleotide changes in 3 of 5 ‘variable’ amplimers result in a single amino acid change (fig. 2). These amino acid changes were not reported previously. All 8 sequenced amplimers were ‘in frame’ and were not interrupted by stop codons.

Real-Time PCR

DNA samples were further analyzed by HTLV-1 *tax*-specific quantitative real-time PCR that provides sensitive detection and allows reproducible quantification of

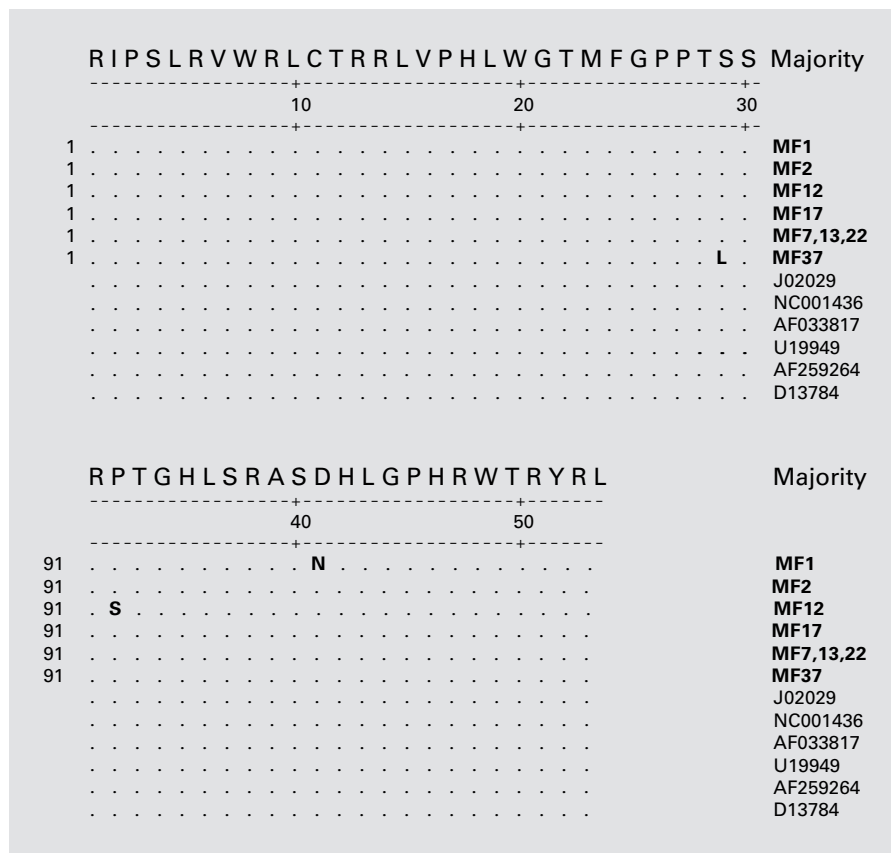


Fig. 2. Alignment of 8 HTLV-1 *tax*-like amplicimers (PCR with SK43-SK44 primers) from blood DNA samples to a panel of HTLV-1 complete genomes (accession numbers are given). Nucleotide sequences of MF7, MF13 and MF22 were identical and homologous to the reference sequences. MF2 and MF17 nucleotide changes result in no amino acid change.

initial copy numbers for a given target sequence. Using dUTP instead of dTTP and uracil-DNA glycosylase treatment, the risk of potential contamination through PCR product carry-over was further reduced. HTLV-1 *tax*-specific sequences were confirmed in 15 blood DNA samples from MF patients by TaqMan PCR. Using quantified HTLV-1 *tax* plasmids as standards, 75,000–150,000 cells were estimated to contain 5–10 copies of HTLV-1 *tax* fragment DNA. All ‘PCR-*tax*’-positive samples were found negative for HTLV-1 *pol* in real-time PCR and PCR/Southern hybridization.

HTLV-1 tax-Like Transcripts in Blood

To analyze possible *in vivo* expression of HTLV-1 *tax*-like sequences, we isolated RNA from whole blood of 3 ‘PCR-*tax*’-positive individuals. Samples were analyzed by RT-PCR followed by Southern hybridization. To exclude RNA contamination with a cellular DNA, analyzed samples were tested in direct PCR reaction without RT step. Positive signals were not detected. *tax* transcripts were revealed in 2 MF patients (fig. 3) indicating transcription of the *tax*-like gene fragment in lymphoid cells

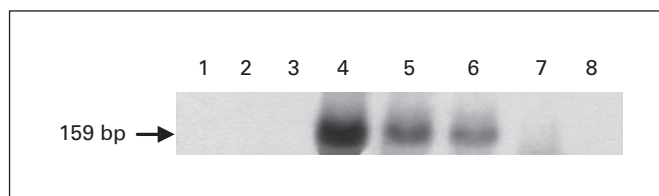


Fig. 3. HTLV-1 *tax*-like transcripts detected by RT-PCR/Southern blot hybridization in ‘PCR-*tax*’-positive blood samples from MF patients. Lines 1–3: direct PCR on RNA specimens from MF patients (negative control); line 4: RNA from MT-2 cells (positive control); lines 5–7: cDNA specimens from MF patients; line 8: H₂O (negative control).

from ‘PCR-*tax*’-positive MF patients. We failed to detect *gag* transcripts in the same individuals.

Antibodies to Tax in Plasma

Using recombinant Tax as an antigen, we performed Western blot analysis of plasma (dilution 1:100) from 8 randomly selected ‘PCR-*tax*’-positive MF patients

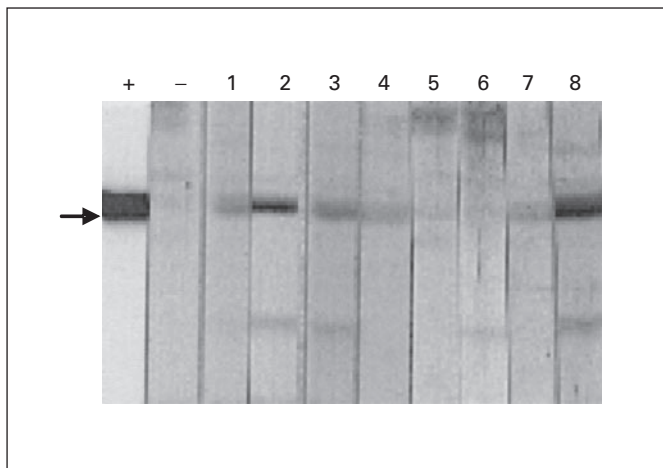


Fig. 4. Antibodies to HTLV-1 p40^{tax} in plasma from 'PCR-tax'-positive MF patients. Lines 1–8: specimens from MF patients; line -: plasma specimen from a healthy blood donor (negative control); line +: monoclonal antibodies (IgG2a) to Tax. Position of the p40^{tax} is indicated by arrow.

(fig. 4). Strong anti-Tax signals were observed in 2 of 8 (No. 2 and 8, respectively) specimens. Four specimens (No. 1, 3, 4 and 7) were weakly positive and 2 (No. 5 and 6) were negative. In addition, we tested 8 randomly selected plasma specimens from healthy blood donors and all 8 were found negative (data not shown). Detection of antibodies to Tax in plasma is an important argument in favor of *tax*-like sequence expression.

No Particle-Associated tax-Like Sequences in Plasma from 'PCR-tax'-Positive MF Patients

A priori, HTLV-1 *tax*-like sequences detected in MF patients might be considered as an integrated part of a genome of a circulating virus either related or unrelated to a primate T cell leukemia virus group. To investigate this possibility, we attempted to isolate a putative virus from plasma of 3 'PCR-tax'-positive individuals using differential centrifugation followed by high-speed centrifugation through a 20% sucrose cushion. Pellets obtained after centrifugation were used for RNA and DNA extraction. RNA and DNA samples were further examined by RT-PCR and direct PCR with SK43-SK44 pair of primers, followed by Southern hybridization. All 3 analyzed samples were negative for HTLV-1 *tax*-like sequences when tested by PCR and RT-PCR. Thus, at the level of sensitivity of the methods used, no particle-associated *tax*-like sequences were detected in 3 'PCR-tax'-positive MF patients.

Phylogenetic Analysis

A 374-bp-long LTR sequence from an MF patient (Kas-MF1, Accession No. AF515451) corresponding to nucleotides 8472–8802 of the ATK sequence was analyzed in a rooted phylogenetic tree using the neighbor-joining method (fig. 5). The analysis of the KAS-MF1 sequence showed that the isolate belonged to the Cosmopolitan HTLV-1 subtype A.

Discussion

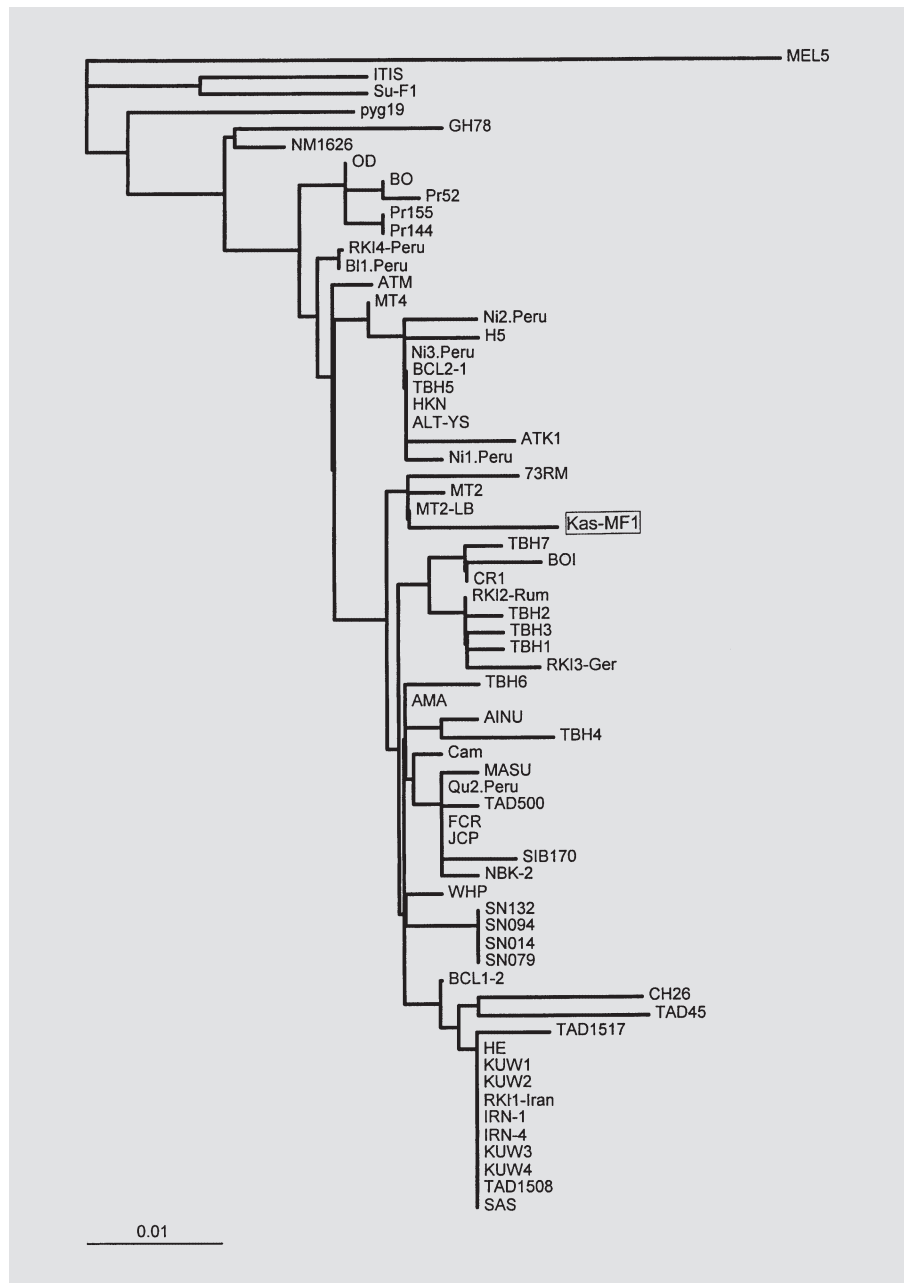
CTCL is a heterogeneous group of malignant T cell lymphomas with primary manifestations in the skin. MF is the most common type of CTCL. Late-stage MF or Sézary syndrome is associated with declining immunocompetence. The mortality rate among MF patients varies widely, largely depending on the stage of disease at diagnosis [22]. Death most often results from systemic infection.

HTLV-1 association with MF is still a matter of controversy. MF patients are HTLV-1 seronegative; however, several groups revealed HTLV-1 markers in MF patients, namely *tax*-like sequences (second exon 159-bp-long fragment) [24, 25–28, 47–49] and HTLV-1 dgs [29, 30]. In addition, HTLV-1-positive cell lines were established from PBMCs of MF patients that were HTLV-1 seropositive [50], seronegative or seroindeterminate [51, 52].

So far, *tax*-like sequences were not detected in MF patients from Europe [53, 54]. Technical difficulties in MF-associated *tax* detection were reported previously [25]. By PCR/Southern hybridization, *tax*-related sequences were revealed in blood DNA of 20 out of 50 (40%) and 3 out of 5 saliva specimens from MF patients. The amount of positive samples was less than that reported by others [28, 47]. However, the investigations mentioned above were carried out in HTLV-1-endemic regions or close to HTLV-1-endemic regions, which may influence the frequency of *tax* detection. Eight of 20 amplicons (DNA from blood) were further analyzed by sequencing. In 8 (80- to 140-bp-long) sequenced fragments, we observed up to 2 nucleotides mismatch. Amplicons were not interrupted by stop codons and were 'in frame' with both *tax* and *rex* genes.

Taken together, these data indicate a possible variability in *tax*-like sequences in MF patients that might not be directly linked to pathogenesis. The sequence variation might be considered as another argument against possible laboratory crosscontamination. By real-time PCR, we confirmed 15 of 20 MF patients to contain HTLV-1 *tax*

Fig. 5. Phylogenetic analysis of the Kas-MF1 LTR sequence. A 374-bp sequence corresponding to nucleotides 8427–8802 of the ATK sequence (accession No. J02029) was compared with HTLV-1 sequences from public data bases. Sequence alignments were analyzed in a rooted phylogenetic tree using the neighbor-joining method. The HTLV-1 subtype C isolate Mel5 was used as an outgroup. The different HTLV-1 subtypes and subgroups are indicated. Kas-MF1 is indicated with a box. The branch lengths are proportional to the evolutionary distance between the analyzed isolates. For a statistical evaluation of the generated tree, a bootstrap analysis was performed using 1,000 bootstrap replicates. The bootstrap values for the major branching points are indicated on the branches in percent.



sequences. It is likely that the other 5 patients contained less than 5–10 copies of the sequence – a detection limit of the real-time PCR in our hands.

Expression of *tax*-like sequences was found by RT-PCR in 2 of 3 ‘PCR-*tax*’-positive individuals, and these individuals were RT-PCR negative for *gag* transcripts. This actually confirms our negative results obtained by direct PCR with HTLV-1 *gag*-specific primers. All of the 60 blood donors, representing our control group, were

negative when tested by PCR with diagnostic HTLV-1 *tax* primers followed by Southern hybridization.

The lack of complete HTLV-1 provirus in MF patients was demonstrated by negative long-range PCR, as well as negative PCR results with *gag*- and *pol*-specific primers. However, by PCR with 5'-LTR-*pX* pairs of primers followed by Southern hybridization, we revealed HTLV-1 dgs in 2 MF patients. One dg was isolated and sequenced. This genome looks as follows: 5'-LTR, 5'-*gag* and 5'-*pX*

region HTLV-1. However, mutations in the first ATG detected in this provirus abolish Gag-like protein synthesis. In a phylogenetic tree analysis, the 5'-LTR fragment of this dg clustered with sequences from the Cosmopolitan HTLV-1a subgroup A.

A rough estimation of the *tax*-like fragment size in MF patients revealed that the fragment is longer than 203 bp, but shorter than 793 bp. Thus, the observed *tax*-like sequence was truncated compared with intact *tax*. Detection of a truncated sequence longer than 159 bp *tax* amplicon argues against contamination with either full-size genomic DNA from MT-2 cells (positive control), or a 159-bp-long amplicon from a previous PCR.

In summary, the following conclusions can be drawn from our data:

(1) Truncated HTLV-1 *tax*-like sequences were detected in blood of 40% of MF patients from the HTLV-1 nonendemic area (European Russia). For the first time, HTLV-1 *tax*-like sequences were revealed in saliva.

(2) Expression of *tax*-like sequences was shown in PBMCs, and antibodies to rp40^{tax} were detected in plasma from 'PCR-*tax*'-positive patients.

(3) No virus particles were detected in plasma specimens from 3 'PCR-*tax*'-positive MF patients.

We did not see any clear association between MF stage and the presence of *tax*-like sequences.

Detection of *tax*-like sequences in saliva might indicate another possible way of the sequence horizontal transmission. We speculate that epithelial cells are most

likely to contribute to saliva '*tax* positivity'; however, we have no data regarding cell type carrying *tax*-like fragments in saliva. In addition, saliva might be considered as an additional biological material to be tested for *tax*, especially in questionable cases, to confirm blood 'PCR-*tax*-positive' data.

In conclusion, detection of few copies of expressing HTLV-1 *tax*-like sequences in MF patients from the HTLV-1 nonendemic region is a bizarre phenomenon that is difficult to explain from the point of view of 'classical virology'. It remains to be determined how *tax*-related sequences are transmitted without a complete HTLV-1 (or another helper virus) and how they may contribute to a disease progression.

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