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ARTICLE

Prevalence of Mycosis Fungoides and Its Association with EBV and HTLV-1 in Pakistanian Patients

Samina NOORALI, Nausheen YAQOOB, Muhammad Israr NASIR, Tariq MOATTER, Shahid PERVEZ

Department of Pathology, The Aga Khan University, Karachi, Pakistan

Mycosis fungoides (MF) is an indolent T cell lymphoma that is distinguished from other lymphomas by its initial appearance on the skin. The histologic diagnosis of MF may be difficult because there is significant overlap in the histologic features of neoplastic T-cell infiltrates and inflammatory dermatoses. This T-cell neoplasm commonly occurs in a mixed, reactive background and can show only a subtle degree of cytologic atypia, rendering histologic diagnosis difficult. In this study MF constituted 0.86% of all non-Hodgkin's lymphoma (NHL) both T and B, as compared to the Western studies which have reported 0.5% prevalence for MF of all NHL. Polymerase chain reaction (PCR) technique was used to assess T-cell clonality in paraffin-embedded skin biopsies clinically and pathologically suspicious for early MF. Out of the 14 cases diagnosed as MF, amplifiable DNA was isolated from 6 cases, which were further studied for T-cell receptor (TcR) – β, γ, and δ chain gene rearrangements. Clonal product was seen in 4 out of 6 cases for β, γ, and δ TcR chain genes. Association for Epstein Barr virus (EBV) was observed in 3 out of 6 cases (50%) of MF. Although these 3 cases were positive for EBV by PCR, but were negative by in-situ hybridization (ISH). No heterogeneity was noted in these 3 cases of MF for BamHI E, K, N, and Z regions of EBV. All six cases were negative for HTLV-1 (tax region) by PCR. It was concluded that the prevalence of MF in Pakistani population is comparable to the Western data, and that EBV association to MF cases was higher than in Western studies. (Pathology Oncology Research Vol 8, No 3, 194–199)

Keywords: Mycosis fungoides (MF), EBV, HTLV-1, *in situ* hybridization (ISH), T-cell receptor (TcR)

Introduction

Mycosis fungoides (MF) is a lymphoproliferative disorder involving neoplastic T lymphocytes. Infiltration of neoplastic cells is invariably demonstrated in the skin. Involvement of the lymph nodes, peripheral blood and viscera is also often found. The histological features of MF are characterised by marked epidermotropism of cytologically atypical T lymphocytes, with convoluted (cereberiform) nuclear contours. These cells form a band like infiltrate in the upper papillary dermis and show formation of Pautrier's microabscesses in the epidermis. There is variable admixture of histiocytes, eosinophils, and plasma cells. Abnormal cells must be present in the epidermis to make a definitive diagnosis of MF.[1] It is estimated that in the USA, MF develops in 1,000 new patients per year and constitutes 0.5% of all NHLs.[2] A clinical diagnosis of MF in its early stages can be difficult and even on histological examination at times is difficult to distinguish it from benign inflammatory dermatoses such as lymphomatoid drug reactions, contact dermatitis, lichen planus, spongiotic dermatitides, pityriasis lichenoides et varioliformis acuta, follicular mucinosis etc, particularly when mixed cell populations with only occasional atypical cells are observed. MF may produce practically all of the patterns used for diagnosing inflammatory skin diseases.[3] MF is characterized immunohistochemically by the presence of

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Correspondence: Dr. Shahid PERVEZ, Department of Pathology, Faculty of Health Sciences, Medical College, The Aga Khan University, Stadium Road, P.O. Box 3500, Karachi 74800, Pakistan. Tel: (92 21) 485-91554/493-0051 Ext.1554, Fax: (92 21) 493-4294, 493-2095, Email: shahid.pervez@aku.edu

(CD 3+) T cell expressing a T-helper/inducer cell immunophenotype (CD4+), with only rare cases having a T-suppressor cell immunophenotype (CD8+).[4] Break points in chromosomes 1,9,14, and 17 and abnormalities involving the gene for the TcR α (14q11) have been noted frequently in patients with MF.[5]

Kanavaros et al[6] and Peris et al[7] have shown no significant role for EBV in the development of cutaneous lymphoma. Whereas Dreno et al have reported 32% prevalence of EBV in cutaneous T-cell lymphomas.[8] Several studies have shown that nearly all patients with MF are HTLV-1 seronegative, but by using polymerase chain reaction (PCR) HTLV-1 DNA sequences has been detected in peripheral blood mononuclear cells and cutaneous lesions of some patients with MF.[9] Other studies have shown no role for HTLV-1 in the development of MF.[10]

The present study is the first to investigate the prevalence of MF, in a large tertiary care referral hospital receiving specimens from all over Pakistan, its association with EBV, HTLV-1 and genetic features to study T-cell receptor (TcR) gene rearrangements.

Materials and Methods

Histology and Immunochemistry

Specimens received in 10% neutral buffered formalin were processed under standardized conditions for paraffin embedding, cut at 5 µm thickness, and stained with hematoxylin and eosin (H & E). Immunohistochemical analysis was performed using CD45 (LCA), CD20 (L26), CD45RO (UCHL1) and CD3 antibodies (Dako, Denmark). Antibody binding was detected by Vectastain (Vector Laboratories Inc, USA) following incubation with specific antibodies. Finally, the slides were counterstained with Harris hematoxylin. Each assay included a negative and a positive control comprising tonsilar tissues.

PCR Amplification of β-globin, EBV and HTLV-1 Genome

DNA from 14 MF patient biopsies, 14 reactive lymph nodes and 30 blood samples was extracted by Nucleon HT kit (Biosciences, USA), according to the manufacturers instruction. Reactive lymph node biopsies and blood samples were used as control for the presence of EBV and HTLV-1. Purified DNA (500 ng) was added to a PCR mix containing 0.2 mM deoxynucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, 150 ng of each primer for β-globin and HTLV-1, 300 ng of each primer for EBV, 2.5U *Taq* DNA polymerase (Advanced Biotechnologies, UK) in a total volume of 50 µl. After initial denaturation at 94°C for 5 mins, 45 cycles of denaturation at 94°C for 1.5 min, annealing 55°C (EBV and β-globin) or 58°C (HTLV-1) for 1.5 min and polymerization 72°C for 1.5 min were carried out in an automated thermal cycler (Perkin Elmer 9600, USA). The cycling program was ended with a final extension at 72°C for 7 minutes. PCR products of 240-bp for β-globin, 239-bp for EBV genome,[11] and 159-bp for HTLV-1 were seen.[12]

In Situ Hybridization (ISH)

Specimens positive for EBV by PCR were further processed for the cellular localization of EBV genome using *in-situ* hybridization (ISH) on fixed tissue sections. Subsequently, tissue sections were hybridized with either biotin labeled EBV, EBER-1 region specific probe or a positive control probe[13] prepared in 20% dextran sulphate solution. A negative control slide was included with each run. The target RNA and probe were denatured at 100C for 5 minutes and hybridized overnight. The hybridized probe was detected using ISH detection system supplied by Life Technologies, USA.

Determination of variation in isolated EBV strains by PCR-SSCP

Heterogeneity in EBV DNA was identified using PCR-SSCP as previously described by Dimitris et al with slight modifications.[14] The *Bam* HI E, N, K and Z regions of EBV were examined for sequence variation. Primers, thermal cycling and product analysis were as reported previously.[15-18] Briefly 5 µl of PCR product was mixed with 5 µl of gel loading dye (95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol) denatured at 95°C for 5 min, cooled on ice and applied immediately to 0.5X mutation detection enhancement (MDE) gel (FMC BioProduct Rockland, USA). Electrophoresis was carried out at room temperature in 0.6X TBE at 280 V. Gels were stained with silver staining technique and photographed.

PCR Analysis of TcR β, γ and δ gene rearrangements

T-cell receptor (TcR) gene rearrangements were studied using β, γ and δ chain primers for the V (variable), D (diversity) and J (junctional) regions for T-cell respectively. Purified DNA (250 ng/µl) was added to PCR mix containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 68 pmoles of primers for β-chain, 50 pmoles of primers for the γ-chain, 30 pmoles of primers for δ-chain and 1.5 U Ampli Taq Gold. Primer sequences, thermal cycling and product analysis were as reported previously.[19-23] Subsequently heteroduplex analysis was performed exactly as described by Langerak et al.[24]

Results

In a period of 10 years between 1992 and 2001 a total of 1610 cases of NHL (both T and B) were diagnosed in the Department of Pathology, The Aga Khan University Hos-

pital, a major referral center in Pakistan. Cutaneous involvement was present in 133 (8.26%), and 14 of such cases (10.5%) were diagnosed as MF on the basis of morphology, immunohistochemistry and where required by TcR gene rearrangement studies. MF contributed 0.86% of the total NHLs. The age ranged between 28 to 73 years with a median age of 52.5 years and a male: female ratio of 2.5:1. Four cases of MF presented as generalized body lesions while the rest exhibited a discrete patch or plaque over different body regions.

Morphology, immunology and molecular characteristics

The cases were diagnosed on the basis of their histological, immunological and molecular characteristics in accordance with the REAL/WHO classification guidelines.[25,26] By immunohistochemistry all 14 cases expressed both T cell markers i.e. UCHL-1 (*Figure 1a,b*) and CD3. These also expressed LCA and were negative for CD20 marker. Out of 14 cases, amplifiable DNA was obtained from 6 cases, which were further studied for TcR gene rearrangements.

EBV and HTLV-1 DNA detection by PCR and ISH

The β-globin PCR was performed on all MF samples out of which amplifiable DNA was obtained in 6 cases. The β-globin PCR positive samples were tested for the presence of EBV DNA and in 3 (50%) samples EBV PCR product of 239-bp was detected. Whereas, no amplifiable product was seen for the tax-region of HTLV-1. The EBV PCR positive samples were also analyzed for the cellular localization of EBV genome using ISH and were found to be negative probably due to low copy number of EBV genome in the cells.

Polymorphism in Bam HI E, K, N, and Z regions by PCR-SSCP

The extent of hyper-variability in the *Bam* HI E, K, N and Z regions of EBV was examined using PCR-SSCP and none of them demonstrated diversity for these regions.

TcR Gene rearrangement

TcR clonality was seen in 4 out of 6 cases, where 2 cases demonstrated both β, γ TcR gene rearrangement, 1 case showed γ, β, δ TcR gene rearrangement and in 1 case only β chain gene rearrangement was seen (*Figure 2a,b,c*).

Discussion

Primary cutaneous lymphomas (NHL) represent a heterogenous group of NHLs, which shows considerable variation in clinical presentation, histology, immunophenotype and prognosis. Approximately 25%-40% of NHLs occur in extra nodal site, skin being the second most common site of extranodal involvement after gastrointestinal tract.[27] MF is an indolent neoplasm of mature helper T lymphocytes that manifests in skin in the form of patches, plaques, tumors or erytheroderma, which may be present on the skin for years and decades. In our study a total of 133 cases of NHLs both B and T presenting as primary skin lesions were diagnosed at AKUH over a span of 10 years (1992-2001). Out of these 133 cases, 14 (10.5%) were diagnosed as MF. In one of the studies MF was diagnosed in adults and the male:female ratio was 2:1.[2] Other study has shown that MF occurs in middle aged adults with median age of 50 years and a M:F ratio of 2.2:1.[28] In our study the mean age of presentation was 50 years, with a median of 52.5 and with the male: female ratio of 2.5:1, which is quite comparable to the Western studies. A study conducted in Tokyo has shown 20.8% prevalence of MF among primary skin NHLs,[29] whereas our study showed 10.5%.

Out of 14 cases of MF, 4 cases (28.5%) presented as generalized body rashes while the rest as discrete localized patches and plaques. One patient presented with

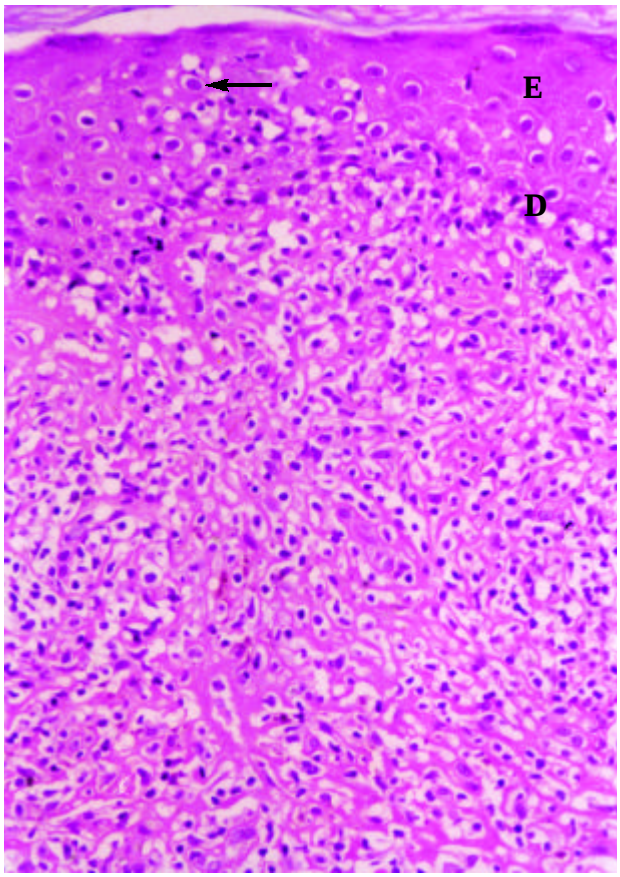


Figure 1a. Photomicrograph of HE stained section of the skin from a patient with MF. Note dense dermal (D) infiltrate with focal epidermotropism (E) by atypical lymphoid cells (Arrow). (20X).

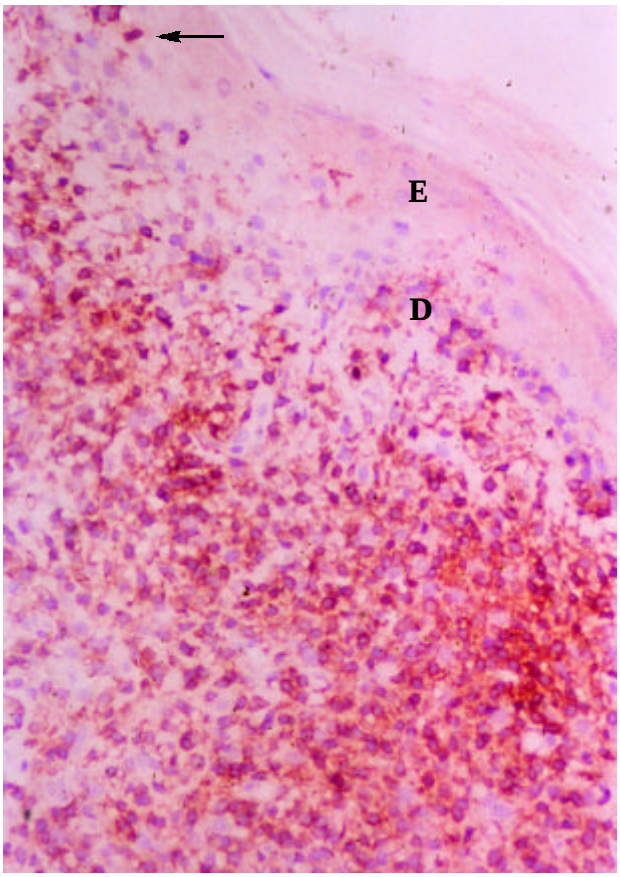


Figure 1b. Serial section from the same lesion stained with a Pan T (UCHL1) antibody. Note strong labeling of atypical lymphoid cells. (40X.)

generalized hyperpigmentation with no raised plaques or tumors. Pigmentary changes in MF are not very rare, although hypopigmented skin lesions are more often reported in literature, there are few cases of MF presenting as hyperpigmented skin lesion.[30] In our study only 1 patient out of 14 presented with flat hyperpigmented diffuse lesion on the trunk.

All MF casess in our study expressed LCA, both T-cell markers i.e., UCHL1 and CD3 and none showed CD20 (a B-cell marker) positivity. Histologically a clear distinction between benign and malignant lymphocytic infiltrates is not always easy. In fact at times it may be impossible to make this distinction purely on morphological grounds. It is precisely here where gene rearrangement studies may be helpful.

The ability to amplify specific regions of DNA from paraffin-embedded tissue by PCR has a profound impact on diagnostic pathology. In our study out of the 14 cases DNA was amplified from 6 cases only. There could be several reasons for the degradation of DNA for example the fixative used, over fixation of tissue in formalin, autolysis, and presence of PCR inhibitors.

Various studies have shown similarities between the skin lesions of MF and Sezary syndrome (SS) and those of Adult T-cell lymphoma/leukemia (ATLL), which is related to HTLV-1. This prompted consideration for a retrovirus role in CTCL. Several studies have shown that nearly all patients with MF are HTLV-1 seronegative, but using PCR, HTLV-1 DNA sequences has been detected in peripheral blood mononuclear cells and cutaneous lesions of some patients with MF.[9] Other studies have shown no role for HTLV-1 in the development of MF.[10] In our study no association between MF and HTLV-1 was observed by PCR.

EBV is a human herpesvirus that infects B-lymphocytes and certain epithelial cells in the oropharynx. It has been frequently identified in cases of nasopharyngeal carcinoma, endemic Burkitt's lymphoma, a subset of cases of Hodgkin's lymphoma and lymphomas developing in the setting of immunosuppression and immunodeficiency.[31,32]

Angel et al[33], have shown no significant association of EBV in 28 cases of primary cutaneous T-cell lymphoma originating from UK. Whereas reports by Dreno et al [8] have shown 32% association of this virus with cutaneous T cell lymphomas, Mouly et al [34] have shown pathogenic role of this virus with cutaneous T-cell lymphoma . Similarly our study has shown association of EBV in 50% cases of MF. All the control samples including reactive lymph nodes and blood samples were negative for the presence of EBV and HTLV-1. Seroprevalence of HTLV-1 in general population was not studied.

Since EBV-infected lymphoid cells and tissues do not exhibit specific morphologic changes, a definitive identification of the virus in tissues requires detection of EBV DNA/RNA or gene products using specialized techniques such as ISH. In our study EBV RNA was not detected by ISH in EBV positive cases by PCR. Similarly a study conducted by Anagnostopolus et al[35] showed presence of EBV in 15 out of 76 cutaneous T-cell lymphomas cases by PCR but with ISH only 7 cases out of 15 showed presence of EBV. Hence above mentioned study and ours indicate that there is a discrepancy between the results obtained by PCR and ISH and is apparently caused by the low copy number of infected cells per tissue section. EBV can be differentiated according to size polymorphism depending on the number of internal repeats in the *Bam* HI E, K, N, and Z regions. We have studied sequence polymorphism in EBV positive cases of MF using SSCP assay in potential hypervariable domains of EBV of *Bam* HI E, K, N, and Z regions in tissue of MF patients. No polymorphism was seen in any of the regions. It is possible that hypervariable loci within the EBV genome are located in *Bam* HI for other regions that were not performed in this study. Whereas Dimitris et al[14] have shown hyper variability in *Bam* HI K and N segments indicating infection by multiple variants of EBV in lymphoid cell lines, tissues, and body fluids.

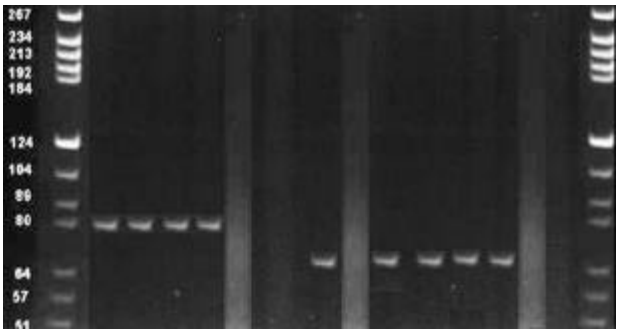
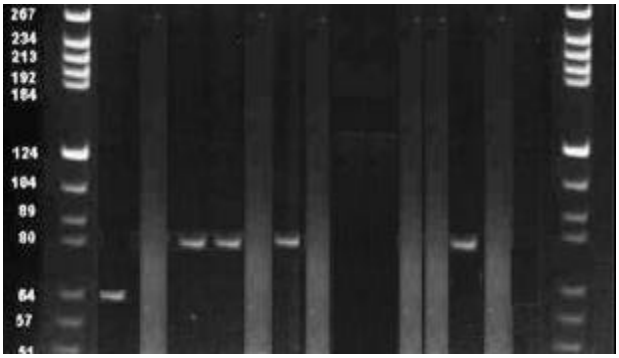
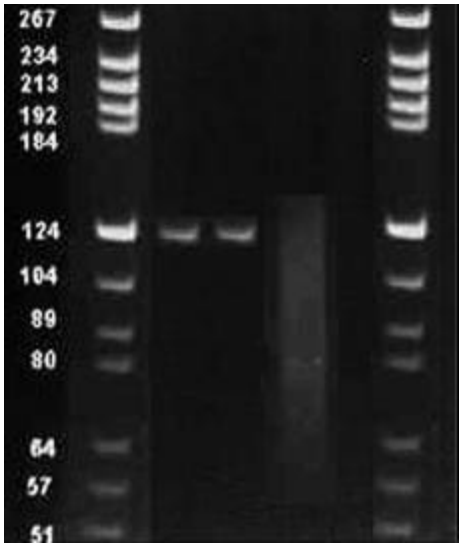


Figure 2. Polyarylamide gels showing TcR β, γ and δ PCR products. Lanes M: pBR322/ HaeIII molecular weight marker, along with cases, positive clonal control (+C), polyclonal control (PC) and negative control (–C). 2a. Sizes of TcR β products for DβJ and of VβJ (50-100 bp) are approximately 80 bp and 70 bp.



2b. TcR g products for VγJ (64-100 bp) and VγJP (80-110 bp) are approximately 80 bp and 70 bp.



2c. Size of TcR δ PCR product is approximately 120 bp.

The T-cell receptor chain gene rearrangements in lymphomas serve as a specific marker for each tumor and detection of such gene rearrangements may enhance the identification of persistent tumor. Molecular analysis of TcR genes is frequently used to prove or exclude clonality and thereby support the diagnosis of suspected T cell proliferations. PCR-based clonality assessment should include analyses that discern between PCR products derived from monoclonal and polyclonal cell populations. One such method is heteroduplex analysis. After denaturation/renaturation, PCR products of clonality rearranged TcR genes give rise to homoduplexes (specific clonal band), whereas in case of polyclonal cells heteroduplexes with heterogeneous junctions are formed (smear).

Ashton et al performed TcR γ-chain gene rearrangement on 27 formalin fixed, paraffin-embedded, clinically and histologically diagnosed MF cases and found clonal product for γ-chain in 16 of 27 (59%) cases.[36] Similarly, study conducted by Toro et al on 3 cutaneous T cell lymphoma (CTCL) cases, showed clonal T-cell receptor γ, δ chain gene rearrangement in all 3 cases[37] having no association with EBV. Whereas in our study we have seen γ, δ TcR chain gene rearrangement in one case with presence of EBV. Straten et al studied TcR β-chain gene rearrangement in 24 CTCL cases comprising of 7 cases of MF, 4 of SS, 3 of lymphomatoid papulosis and 3 of large cell CTCL with CD30 expression and 7 without CD30 expression. Clonal product was seen for β-chain in 18 patients, whereas in remaining 6 no clonal product was seen.[38] In our study out of the 6 cases clonal product was seen for TcR β, γ, and δ chain genes in 4 cases out which 3 cases showed EBV presence. The remaining 2 cases showed polyclonal smear suggesting that β, γ chain gene rearrangement is also common among MF patients in our population. Besides this, we have also seen TcR δ-chain gene rearrangement in 1 patient (*Figure 2a,b,c*). In addition, this study further affirms that the detection of rearranged T-cell receptor genes can be a sensitive and practical method for the diagnosis and characterization of T-cell neoplasm.

Hence we conclude prevalence of MF in Pakistani population is comparable to Western studies, higher frequency of EBV may contribute to the pathogenesis of the disease and γ, β TcR clonal gene rearrangements are common in MF.

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