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Increased Twist expression in advanced stage of Mycosis fungoides and Sézary syndrome

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Abstract

Background—The mechanisms of tumor progression in mycosis fungoides (MF) and Sézary syndrome (SS) are poorly understood. Twist, a transcription factor, is thought to promote solid tumor progression by blocking p53 and inhibiting c-myc-induced apoptosis. Whether Twist expression is correlated to MF/SS stages remains unknown.

Methods—Twist, c-myc and p53 proteins in 68 MF/SS lesions across all T stages were examined by immunohistochemistry, and mRNA levels in peripheral blood CD4+ T-cells from SS patients were measured by QT-PCR.

Results—Positive staining for Twist was found in 12.5% (2/16) of T1 and 33.3% (7/21) of T2 early stage patches/plaques compared to 50.0% (9/18) of T3 tumors and 84.6% (11/13) of T4 erythroderma. Most T4 erythroderma were positive for Twist in dermal lymphocytes, with the strongest staining. Positive staining for c-myc was higher in T3/T4 lesions (29/31, 93.5%) than T1/T2 lesions (25/37, 67.6%, $p<0.05$), with strongest staining in T3 tumors. Aberrant p53 expression was more common in T3/T4 lesions (8/31; 25.8%) than in T1/T2 lesions (2/37, 5.4%, $p<0.05$). Twist mRNA was detected in all CD4+ T cells from SS patients but not in normal donors.

Conclusions—Increased Twist protein expression in advanced MF/SS lesions suggests that Twist expression may correlate with MF/SS stages.

Keywords

Mycosis Fungoides; Sézary Syndrome; Twist; c-myc; p53; cutaneous T-cell lymphoma

Introduction

Cutaneous T-cell lymphoma (CTCL) represents extranodal non-Hodgkin lymphoma characterized by malignant clonal proliferation of skin-homing CD4+CD45RO+ helper/memory T cells often with associated loss of CD7 and CD26 expression (1–3). Mycosis fungoides (MF), the most common form of CTCL, has an indolent course, but about 34% of patients will progress to advanced stages of disease, i.e. transformed tumors, nodal involvement, or peripheral blood involvement, with an unfavorable prognosis (4). Sézary syndrome (SS), a leukemic form of CTCL, can arise *de novo* or evolve from MF with a poorer prognosis. The median overall survival was 5.4 years for patients with 1000 to 10,000 Sézary cells/ μ l and only 2.4 years for patients with >10,000 Sézary cells/ μ l in our cohort of erythrodermic patients (5).

Loss of antigen-driven, activation-induced cell death through the Fas/Fas ligand pathway may initiate the accumulation of memory T-cells (6–8), but the molecular mechanisms underlying tumor transformation and progression are poorly understood. Although new treatments have become available, few are able to induce durable complete remissions (9). Understanding molecular mechanisms underlying progression of early MF/SS to advanced stage MF/SS may translate into useful biomarkers for predicting disease progression and for developing more specific targeted therapies.

Twist protein (also called Twist 1) is a highly conserved transcription factor that belongs to the family of basic helix–loop–helix (bHLH) proteins and functions as a regulator of the epithelial-mesenchymal transition (EMT) during embryogenesis (10). Twist is retained in adult mesodermal-derived tissues but is normally not expressed in lymphoid cells (11, 12). Studies have found that inactivation of Twist in animal models induces massive apoptosis during mammalian development (13). *In vitro* experiments in mouse embryo fibroblasts showed that Twist blocked the c-myc-induced apoptotic response through modulation of the ARF/MDM2/p53 pathway (14). Twist has been reported to be over-expressed in several human solid tumors; including breast cancer, prostate cancer, gastric carcinoma, and neuroblastoma (15–18), and elevated Twist protein levels are associated with advanced tumor stage and poor prognosis (19–21). Recently, Cosset et al found that Twist was up-regulated during chronic myeloid leukemia (CML) progression and in imatinib-resistant CML cells (22). Several members of bHLH family (TAL1, TAL2, and LYL1) have been found to be T-cell onco-proteins (23).

Twist abnormality was first reported by Van Doorn et al as aberrant expression of Twist in Sézary syndrome, which was identified by gene expression analysis (24). Our group later confirmed that Twist mRNA is highly expressed in Sézary cells (25). Comparative genomic hybridization (CGH) analysis of tumor DNA from MF/SS patients conducted by Vermeer et al and us (unpublished data) has revealed a gain of a chromosomal region on 7p21.1 that harbors the Twist gene in 45% of MF patients and 20% of SS patients (26). A gain of 8q24 (harboring the myc oncogene) and a loss of 17p13 (harboring the TP53 tumor suppressor gene) were also found in MF and SS patients with different incidences (23% and 9% in MF vs. 75% and 70% in SS, respectively (27).

Since little is known about Twist expression in MF/SS tissues, identifying Twist in MF/SS tissues may allow us to further study the role of Twist in MF/SS disease progression. We hypothesized that Twist expression is correlated to MF/SS stages and may cooperate with c-myc and p53 in disease progression. To test this hypothesis, we evaluated Twist, c-myc, and p53 protein expression in MF/SS lesions across all T stages by immunohistochemistry. We also quantified Twist, c-myc, and p53 mRNA in peripheral blood CD4⁺ T cells from SS patients by real-time quantitative PCR (QT-PCR).

Materials and Methods

Tissue and blood specimens

The study was performed in accordance with the Declaration of Helsinki. All patients signed Institutional Review Board approved consent forms. Lesional skin biopsies with different T stages (1–4) were taken from 68 newly diagnosed MF/SS patients at The University of Texas MD Anderson Cancer Center. The 6-mm punch biopsies were fixed in 4% paraformaldehyde and stored in 80% ethanol until paraffin embedding. Normal healthy donor (HD, n=3), psoriasis (PS, n=3), and squamous cell carcinoma (SCC, n=3) skin specimens were used as controls. The fresh peripheral blood was drawn from 5 patients with SS and peripheral blood mononuclear cells (PBMC) were isolated. Healthy donor blood samples were received from Department of Transfusion Medicine of our institution.

Immunohistochemistry

The consecutive sections (5- μ m) from each tissue paraffin block were cut, and duplicate sections from 3 blocks were put in a single slide. Twenty-three slides were constructed from 68 tissue blocks, creating a modified tissue array. Immunohistochemical staining was performed with polyclonal rabbit anti-Twist (H-81, 1:50, Santa Cruz Biotechnology Inc. Santa Cruz, CA), monoclonal mouse anti-c-myc p67 (9E10, 1:10, eBioscience, Inc. San Diego, CA), and anti-p53 (DO-7, 1:50, Dako North America, Inc. Carpinteria, CA) antibodies using the Envision system (Dako North America). DAB+ substrate-chromogen resulted in a brown precipitate at antigen sites. Tissue slides incubated with rabbit IgG fraction and mouse IgG1 served as negative controls. Squamous cell carcinoma was served as positive controls for p53 and c-myc staining. Psoriatic skin lesions were used as inflammatory disease controls for Twist. The semi-quantitative analysis was used to evaluate positive staining as previously described (28). The positive staining for Twist and c-myc by lymphocytes was graded for the intensity and percentage cells (%) separately (MD, XN). In detail, staining intensity was graded as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3). The percentages (%) of positive cells in the dermal lymphocytic infiltrate was determined under high magnification in three high power fields ($\times 200$) (28). The p53 staining was interpreted as either positive or negative.

Real-time quantitative PCR (QT-PCR)

CD4⁺ T cells were selected using T Cell Isolation Kit (Miltenyi Biotec, Germany) from PBMCs of 5 SS patients and 5 healthy donors. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA). First strand cDNA was synthesized with an oligo (dT) 12–18 primer using SuperScript III reverse transcriptase (Life Technologies Inc., Gaithersburg, MD). The QT-PCR was used to measure relative levels of Twist, c-myc and p53 mRNA with the ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc. Foster City, CA). Pre-formulated TaqMan primers and probes were used, and relative levels of gene expression were normalized to GAPDH (25).

Statistical analysis

Statistical significance of gene expression levels, intensities, and distribution of positive staining across tumor stages for each gene tested was assessed using Fisher's exact test, student t-test, and one-way ANOVA. Differences between experimental groups were considered significant if $p < 0.05$. Statistical analyses were conducted using SAS 9.2 for Windows (Copyright @ 2011 by SAS Institute Inc., Cary, NC) and Microsoft Office Excel 2007.

Results

Patients and lesions

The demographics of 68 MF/SS patients studied in the tissue array are summarized in Table 1. The median age was 56.4 years (range 18–83), with 28 females and 40 males. These lesions were stratified into 4 skin stages (T1–T4) according to the ISCL/EORTC revised criteria for MF and SS (29). They included 16 T1 stage lesions defined as limited patches/plaques covering <10% of the body, 21 T2 stage lesions defined as patches/plaques covering 10% of the skin surface, 18 T3 stage lesions defined as one or more tumors (≥ 1 cm diameter), and 13 T4 stage lesions from patients with erythema covering ≥ 80 % body surface area. The T4 lesions consisted of 6 with erythrodermic MF and 7 with SS. For the purpose of analysis, we also grouped T1 and T2 lesions as early stage lesions and T3 and T4 lesions as advanced stage lesions. Large cell transformation was defined as lesions in which >25% of the malignant cells had nuclei four times larger than normal (30). Large cell

transformation was present more frequently in T3/T4 advanced stage lesions (5/31, 16.1 %) than in T1/T2 early stage lesions (1/37, 2.7%). Squamous cell carcinoma (SCC, 1/68 in T3, 1.5%) and basal cell carcinoma (BCC, 1/68 in T4, 1.5%) were occasionally found in patients with T3/T4 advanced stage lesions. Five SS patients enrolled for blood study were all stage IV, and 4 of 5 were new untreated SS patients whose blood contained > 85% circulating CD4+/CD26(-) T cells. The fifth SS patient was treated with Vorinostat for 7 months and had only 18.1% circulating CD4+/CD26(-) T-cells.

Twist Expression in MF/SS

To determine whether Twist expression is correlated to MF/SS T stage, we examined Twist protein expression across all stage MF/SS lesions by immunohistochemistry. As summarized in Table 2, the percentages of MF/SS lesions positive for Twist in dermal lymphocytes were 42.6 % (29/68). Positive staining for Twist was found in 12.5% (2/16) of T1 and 33.3% (7/21) of T2 early stage patches/plaques compared to 50.0% (9/18) of T3 tumors and 84.6% (11/13) of T4 erythroderma, respectively ($p<0.01$, Fisher's Exact). More T3/T4 advanced stage lesions expressed Twist (20/31; 64.5 %) than T1/T2 early stage lesions (9/37, 24.3 %, $p=0.0013$). Most T4 erythrodermic lesions were positive for Twist (11/13, 84.6%), compared to T1/T2/T3 lesions (18/55, 32.7 %, $P=0.0011$). Positive staining for Twist was seen in small subsets of dermal lymphocytic infiltrates in lesions of all stages, but more positive cells were observed in T3/T4 advanced stage lesions compared to T1/T2 early stage lesions (Figure 1A and Figure 2). Few or no epidermal lymphocytes were positive for Twist. Intensity of staining for Twist was also stronger in T3/T4 advanced stage lesions (1.34 ± 1.44) compared to 0.36 ± 0.54 in T1/T2 early stage lesions ($p<0.001$). Notably, the strongest staining was present in T4 erythroderma (1.76 ± 0.98) compared to discrete T1/T2/T3 skin lesions (0.58 ± 0.96 , $p<0.001$). Strong staining for Twist was also seen in fibroblasts in some T4 lesions (Figure 2D). There was little to no staining seen in three psoriatic lesions tested (Figure 2A). In summary, positive staining for Twist were present in dermal lymphocytes in all T stage MF/SS lesions, with higher frequency and intensity present in late stage MF/SS lesions, especially in T4 erythrodermic lesions.

As shown in Figure 3A, Twist mRNA was detected in all CD4+ T cells from SS patients by QT-PCR ($n=5$, 3.34 ± 5.72 , $p<0.05$). Levels of Twist mRNA varied (0.02 ~ 13.31), with higher levels present in four patients with higher percentages of circulating CD4+/CD26(-) T cells and the lowest in one patient with lowest numbers of CD4+/CD26(-) T cells. Of note, Twist mRNA was undetectable in CD4+ T cells from all normal donors except one with a very low level of Twist mRNA ($n=5$, 0.01 ± 0.01).

Expression of c-myc in MF/SS

To determine whether c-myc expression is also correlated to MF/SS T stage, we assessed c-myc protein expression in the same tissue array by immunohistochemistry. As summarized in Table 2, there were 79.4% of MF/SS lesions positive for c-myc (54/68). Positive c-myc staining was seen in 68.8 % (11/14) of T1, 66.7 % (14/21) of T2, 94.4 % (17/18) of T3, and 92.3 % (12/13) of T4 lesions, respectively. The majority of T3/T4 advanced stage lesions expressed c-myc (29/31, 93.5%) compared to only two thirds of T1/T2 early stage lesions (25/37, 67.6%, $p=0.0141$). As shown at Figure 1B and Figure 4, more dermal lymphocytes were positive for c-myc in T3/T4 advanced stage lesions (15.8 ± 1.6 %) than in T1/T2 early stage lesions (7.6 ± 2.9 %, $p<0.05$). T3 tumor lesions were highly positive for c-myc, with more positive cells and higher intensity than T1/T2 early stage lesions ($p<0.001$). All T3/T4 advanced stage lesions staining positive for Twist were also positive for c-myc (20/20, 100 %), and 7 of 9 (77.8%) T1/T2 early stage lesions staining positive for Twist were positive for c-myc. Therefore, positive staining for c-myc was present in dermal lymphocytes in all T

stage MF/SS lesions with higher frequency and intensity present in late stage MF/SS lesions, especially in T3 tumor lesions.

QT-PCR analysis showed that c-myc mRNA was significantly higher in CD4+ T cells from SS patients (25.13 ± 14.77 ; $p < 0.05$) than in CD4+ T cells from normal donors (5.67 ± 3.39) (Figure 3B).

Expression of p53 in MF/SS

Since Twist may block the c-myc-induced apoptosis by inhibiting p53 function (14), we further examined p53 protein in the MF/SS tissue array by immunohistochemistry. It is known that only mutant p53 with a prolonged half life can be detected by immunohistochemical staining (31). As summarized in Table 2, by immunohistochemistry, 14.7% of MF/SS lesions were positive for p53 (10/68), including 12.5 % (2/16) of T1, 0% of T2, 33.3 % (6/18) of T3, and 15.4% (2/13) of T4 lesions ($p < 0.05$, Fisher's Exact). More lesions in T3/T4 advanced stage lesions (8/31, 25.8%) were positive for p53 than T1/T2 early stage lesions (2/37, 5.4%, $p < 0.05$), and T3 tumor lesions had highest incidence of p53 staining (Table 2 & Figure 5). All 10 lesions positive for p53 were also positive for c-myc. Four of 20 T3/T4 advanced stage lesions positive for Twist were also positive for p53, but none of 9 T1/T2 early stage lesions positive for Twist were positive for p53. Thus, aberrant p53 was present more often in advanced stage lesions, especially in T3 tumor lesions.

The level of p53 mRNA in CD4+ T cells from SS patients (0.81 ± 0.34) by QT-PCR was much lower than that from normal donors (147.68 ± 115.64 ; $p < 0.05$, Figure 3C). This result indicates that there was a decrease/suppression of wild type p53 mRNA in SS patients.

Discussion

Our findings of increased Twist protein expression in advanced MF/SS lesions and high levels of Twist mRNA in peripheral blood CD4+ T cells from SS patients suggest that Twist expression is correlated to MF/SS stages. The combination of increased Twist and c-myc expression with abnormal p53 expression support further investigation of the role of Twist in the progression of MF/SS and the cooperation between Twist, p53 and c-myc in MF/SS.

Our results confirm the findings of high levels of Twist mRNA in patients with SS (24, 25). Furthermore, Vermeer et al, van Doorn et al, and our group (unpublished data) also found increased copy numbers or trisomy of the Twist locus in patients with SS (24–27). Through quantitative analysis, we found high variability of Twist expression between MF/SS patients, suggesting a heterogeneous, complicated pathogenesis in MF/SS. Our data are first to show that expression of Twist protein increases in frequency and intensity in advanced stage MF/SS lesions. Although the abnormal expression of c-myc and p53 in human CTCL cell lines and MF/SS lesions has been previously studied, the relationship between c-myc, p53 and Twist was not formerly appreciated (32–35).

As mentioned before, wild type p53 protein cannot normally be detected due to its short half life. Many p53 gene mutations result in expression of protein with a prolonged half life resulting in cellular accumulation and detection by immunohistochemical staining (31). Our study shows that T3 tumor lesions had highest incidence of mutant p53, which is consistent with McGregor's previous study (35) and supports the conclusion that p53 mutation does play a key role during the disease progression of MF/SS. However, our finding of abnormal p53 expression in less than 33.3 % of advanced MF/SS lesions suggests that besides p53 mutations, other alternative events, such as Twist over-expression, could inhibit p53 function and contribute to lymphomagenesis(34). Further investigation with bigger sample size is needed to confirm this finding.

MF and SS have recently been re-classified as distinct entities by Campbell et al, and they suggested that SS and MF arise from distinct memory T-cell subsets (36). They propose that SS is a malignancy of central memory T cells (T_{CM}) and MF is a malignancy of skin resident effector memory T cells (T_{EM}), and this could explain their distinct clinical behavior (36). A recent study done in mice and humans reported that normal T helper (Th) 1 effector memory cells transiently increased Twist expression after activation, while Th2 effector memory cells and T_{CM} did not (37). This phenomenon could be seen in chronic inflammatory diseases (37). In our study, Twist staining was not present in control psoriatic lesions and was very limited in early stage MF lesions but was highest in advanced T3/T4 lesions. In keeping with the presence of high mRNA levels in SS cells, almost 90% of erythrodermic T4 lesions expressed Twist protein. Further studies to determine which cell types (CD4, CD8, T_{CM} or T_{EM} , and Th1 or Th2) are positive for Twist in MF/SS lesion are needed.

STAT3, a transcription factor found on chromosome 17q, is known to be constitutively activated in CTCL cell lines and MF lesions and can activate NF- κ B signaling and proliferation (38–40). STAT3 directly binds to the second proximal STAT3-binding site on the human Twist promoter, it could in turn up-regulate Twist and activate its transcriptional activity in human tumors (19, 20). Cheng et al. found that inhibiting STAT3 expression with a small molecule inhibitor, JSI-124, resulted in significant reduction of Twist protein and mRNA expression (19). Inhibition of STAT3 also induced apoptosis of SS derived Hut78 cells (41), and we speculate that this could be the case in MF and SS.

Although several oncogenic alterations have been reported in MF and SS (42), there are no common mutations or tumor biomarkers which explain the pathogenesis. Recent studies found that Twist 2, another member of Twist family, was also over-expressed in mature T-cell lymphoma cells and could down-regulate CD7, a galectin-1 receptor. This may in turn decrease galectin-1-mediated apoptosis of activated T-cells, promote malignant T-cell accumulation, and progression of T-lymphoma (43). We believe that further elucidation of the role of Twist in MF and SS may facilitate better understanding of the mechanisms of tumor transformation and progression of MF and SS, and represent potential therapeutic targets.

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Figure 1. The prevalence of Twist and c-myc protein expression in MF/SS lesions by immunohistochemistry

A. The positive staining for Twist by dermal lymphocytic infiltrates (intensity and %) in T1, T2, T3, and T4 MF/SS lesions; **B.** The positive staining for c-myc by dermal lymphocytic infiltrates (intensity and %)) in T1, T2, T3, and T4 MF/SS lesions.

Figure 2. Twist protein expression in MF/SS lesions by immunohistochemistry
Twist protein was examined using polyclonal rabbit anti-Twist (H-81, 1:50) in psoriatic lesions (**A**), T1 MF patches (**B**), T3 MF tumors (**C**); and T4 erythrodermic lesions (**D**). Black arrows indicate positive dermal lymphocytes or fibroblasts. $\times 320$

Figure 3. The mRNA levels of Twist, c-myc, and p53 in CD4+ T cells from SS patients and healthy donors

Total RNA was extracted from peripheral blood CD4+ T cells of SS patients and healthy donors (HD). First strand cDNA was synthesized with an oligo (dT) 12–18 primer. QT-PCR was used to measure relative levels of Twist (A), c-myc (B), and p53 (C)mRNA, and relative fold changes of gene expression were normalized to GAPDH.

Figure 4. C-myc protein expression in MF/SS lesions by immunohistochemistry

Expression of c-myc protein was examined using monoclonal mouse anti-c-myc p67 (9E10, 1:10) in the normal skin (A), T1 MF patch (B), T3 MF tumor (C), and T4 erythrodermic lesions (D). $\times 200$

Figure 5. P53 protein expression in MF/SS lesions by immunohistochemistry

Expression of p53 protein was examined using monoclonal mouse anti-p53 (DO-7, 1:50) in the normal skin (A), T1 MF patches (B), T3 MF tumor lesions negative for p53 (C); and T3 MF tumor lesions positive for p53 (D). $\times 200$

Table 1

Demographics of MF and SS patients and staging

Tumor Stage	Sample size	Age (years)	Sex (F/M)	Diagnoses and disease stage
T1	16	54.9 (30–79)	6/10	MF IA (n=15);MF IVA with LCT (n=1)
T2	21	49.2 (18–79)	8/13	MF IB (n=17);MF IIA (n=2);MF IIB (n=1);MF IVA (n=1)
T3	18	61.3 (25–81)	6/12	MF IIB (n=10);MF IIB with SCC ^{**} (n=1); MF IIB with LCT (n=2);MF IVA (n=1);MF IVB (n=2);MF IVB with LCT (n=2)
T4	13	63.0 (43–83)	8/5	MF III (n=1);MF IVA (n=2);MF/SS ^γ IVA with LCT (n=1);MF IVB (n=3);MF/SS IVB (n=5);MF/SS IVA with BCC ^{***} (n=1)
Total	68	56.4 (18–83)	28/40	MF (n=61); SS (n=7)

* Large cell transformation;

** Squamous cell carcinoma;

^γ Patients with SS evolving from MF;

*** Basal cell carcinoma

Table 2

The prevalence of Twist, c-myc, and p53 protein expression in MF/SS lesions by immunohistochemistry

	T1 (n=16)	T2 (n=21)	T3 (n=18)	T4 (n=13)	Total (n=68)	Fisher's Exact p-value
Twist	12.5 % (2)	33.3 % (7)	50.0 % (9)	84.6 % (11)	42.6 % (29/68)	0.0007
c-myc	68.8 % (11)	66.7 % (14)	94.4 % (17)	92.3 % (12)	79.4 % (54/68)	0.0690
p53	12.5 % (2)	0 % (0)	33.3 % (6)	15.4 % (2)	14.7 % (10/68)	0.0193