Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Transcriptional Profiles Predict Disease Outcome in Patients with Cutaneous T-Cell Lymphoma

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Abstract

Purpose: Average survival of cutaneous T-cell lymphoma (CTCL) is associated with clinical stage at diagnosis, where stage I has a favorable survival prognosis, whereas patients with more advanced stages succumb to their disease within 5 years. Although the majority of patients present with an early-stage CTCL, 15% to 20% of them will inevitably progress. Current state-of-the-art clinical criteria cannot identify individuals with stage I disease who are at risk of progression. The purpose of the current work is to gain novel molecular insight into the pathophysiology of CTCL to be able to identify patients with poor versus favorable prognosis. Our previous work used microarray analysis of skin biopsies from 62 CTCL patients to perform an unsupervised analysis of gene expression, which revealed three distinct transcription profile clusters.

Experimental Design: In the present study, we used reverse transcription-PCR to confirm gene expression levels for a subset of representative genes in each cluster. We also performed a Kaplan-Meier analysis of survival and disease progression based on the 6 years of clinical follow-up.

Results: Our reverse transcription-PCR results confirmed the upregulation of representative genes for each cluster, whereas clinical analysis documents that all stage I cases that progressed to stage II and beyond were in poor and intermediate prognosis clusters 1 and 3 and none were in favorable prognosis cluster 2. This analysis also identified certain genes that were preferentially expressed in favorable (e.g., *WIF-1*) versus poor (e.g., *IL-17F*) prognosis clusters.

Conclusion: This work suggests that it may be possible to stratify CTCL patients into low-risk, intermediate-risk, and high-risk groups based on gene expression. *Clin Cancer Res; 16(7); 2106–14. ©2010 AACR.*

Cutaneous T-cell lymphoma (CTCL) is a term formally adopted in 1979 to describe a group of lymphoproliferative disorders characterized by localization of neoplastic T lymphocytes to the skin (1). CTCL represents a heterogeneous group of non–Hodgkin lymphomas, with mycosis fungoides and its leukemic variant Sezary syndrome being the most common forms. Approximately 16,000 to 20,000 individuals are affected by CTCL in the United States, with ~1,500 new cases being reported each year (2–4). The incidence of CTCL is increasing, with one study from 1973 to 2002 documenting a 3.4-fold increase during the course of the study (2). This disease primarily affects individuals who are >50 years of age, with a slight predilection for black and/or male individuals (2, 5). The molecular pathogenesis of CTCL remains unknown.

Average survival after the diagnosis of CTCL is associated with the clinical stage at time of diagnosis. Early-stage (i.e., stage IA and IB) disease has an indolent course, with normal or near-normal life expectancy in stage IA disease and a favorable 5-year survival rate (i.e., ~100% for stage IA and 95% for stage IB; refs. 6, 7). In contrast, more advanced stages of CTCL are associated with recalcitrant disease and poor 5-year survival rates (Table 1). In particular, stage IV Sezary syndrome disease has a 5-year survival rate ranging from 30% to 51% (6–8). Most CTCL patients present with an early-stage (i.e., IA or IB) disease (6, 7, 9). However, approximately 10% to 20% of these patients will have a progressive and fatal clinical disease, whereas the rest will have a favorable survival and will experience an indolent course (6, 7, 9). Although most patients with an advanced CTCL will have a poor prognosis, a minority of these patients will survive for much longer than 5 years. Early identification of patients at risk of progression would allow for earlier use of more aggressive therapies. Unfortunately, currently there are no molecular/biological markers available to predict which patients with early-stage CTCL

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Translational Relevance

In most cases, early-stage cutaneous T-cell lymphoma (CTCL) patients experience an indolent disease course with a favorable prognosis. However, 15% to 20% of stage I disease patients will progress to higher stages that are associated with significant morbidity and mortality. Unfortunately, it is not possible to predict based on the available clinical criteria which individuals are likely to progress. In the current study, we validate three distinct genetic signature patterns that can help segregate patients into low, intermediate, and high risk of progression clusters. The current work suggests that it may be possible to use a select group of genes to identify stage I CTCL patients with poor disease prognosis. Such stratification may enable clinicians to effectively detect and treat high-risk CTCL at an early stage while sparing the patients with low risk/indolent disease from exposure to toxic and expensive medications.

will progress or which patients with an advanced disease will enjoy a longer than expected life.

To elucidate such prognostic molecular markers and to gain additional insight in disease etiology, we previously performed a microarray gene expression analysis of skin samples from 62 CTCL patients who presented with various clinical stages at the time of biopsy and diagnosis (Table 2; ref. 10). This work revealed three distinct transcription profile clusters (i.e., clusters 1, 2, and 3), where clusters 1 and 3 contained a mix of stage I to IV disease patients, whereas cluster 2 contained mostly stage I and only a few cases of advanced-disease patients (Table 2; ref. 10). It is notable that stage I disease patients were well represented in each cluster (i.e., cluster 1 had 11, cluster 2 had 18, and cluster 3 had 14 stage I patients; Table 2). In fact, 42 of 62 (68%) of all patients in the study had stage I disease at the time of diagnosis. The described three distinct transcription profile clusters were associated with different clinical courses. During the initial 3-year follow-up, cluster 2 genes associated with the best clinical outcome and good response to therapy, whereas cluster 1 and 3 genes associated with the worst and intermediate clinical outcomes, respectively, and poor response to therapy (10).

In the present study, we used reverse transcription-PCR (RT-PCR) analysis to confirm gene expression findings in the three cluster models derived from the microarray study. We further correlated the obtained molecular data with an additional 3 years of clinical follow-up. These findings confirm the previously observed clinical trends and show that all of the cases of stage I CTCL that progressed to stage II or greater disease were in clusters 1 and 3 and none were in cluster 2. We also used an RT-PCR expression analysis to identify specific genes that are preferentially expressed in favorable versus poor prognosis patients.

Patients, Materials, and Methods

Patients and samples. All patients were enrolled in an Institutional Review Board–approved study protocol with informed consent. Patients were recruited from the Cutaneous Lymphoma Clinic at the Dana-Farber Cancer Institute/Brigham and Women's Hospital. An extensive chart review was conducted for all patients to collect information on clinical parameters and outcomes between January 2003 and January 2009. Data were stored in a study database. All tissue samples were obtained and processed as previously described (10). Briefly, 6-mm punch biopsies from involved skin were collected from 62 patients between January 26, 2003 and June 1, 2005.

The obtained 6-mm biopsies were immediately snap-frozen in liquid nitrogen. Tissue was powdered in liquid nitrogen (Cryo-Press, Microtec Co.), and total RNA was extracted using Trizol (Invitrogen, Inc.) and converted to cDNA using the iScript RT-PCR kit (Bio-Rad) according to the manufacturers' instructions. The biopsy samples analyzed in this report are the same samples that were analyzed by microarray in the previous study (10).

Quantitative real-time RT-PCR gene expression analysis. A set of genes that were most strongly upregulated in each of the three clusters were selected for confirmation by RT-PCR analysis. Primers for candidate human genes were designed using Primer 3 web software (11) and were purchased from Invitrogen. RT-PCR was done using the obtained cDNA from CTCL patients and iScript RT-PCR mix (Bio-Rad) on a Bio-Rad iCycler as previously

Table 1. CTCL clinical disease stage survival rates adopted from Refs. [7, 8]

Clinical stage	5-y disease-specific survival (%)	15-y disease-specific survival (%)	Mean survival (y)
IA	96	98	Normal life expectancy
IB	95	85	12.9
IIA	84	71	
IIB	56	32	4
III	65	49	
IV	30-51	14	1.5-5.1

Table 2. Clinical stages of CTCL patients enrolled in the study

CTCL stage	No. of patients
I	43
II	4
III	6
IV	9
Total	62

Cluster 1	
CTCL stage	No. of patients
I	11
II	2
III	2
IV	5
Total	20

Cluster 2	
CTCL stage	No. of patients
I	18
II	1
III	1
IV	0
Total	20

Cluster 3	
CTCL stage	No. of patients
I	14
II	1
III	3
IV	4
Total	22

described (12). The obtained data were analyzed using the XLSTAT 2009 software to obtain Kaplan-Meier curves (13). *P* values were calculated using the log-rank test (14).

Results

RT-PCR expression analysis confirms three distinct molecular CTCL signatures. Whereas microarray analysis provides an unprecedented capacity for whole genome expression profiling, it has a number of inherent pitfalls that have been described elsewhere (15–17). Quantitative real-time PCR (RT-PCR) is a commonly used validation tool for confirming gene expression results obtained from microarray analysis and currently serves as the "gold standard" approach for evaluation of gene expression (16). Thus, we wanted to confirm our previous microarray findings using RT-PCR technology. More than 25 genes from all three clusters (i.e., poor prognosis cluster 1, favorable prognosis cluster 2, and intermediate prognosis cluster 3) were selected based on the previous microarray results. Expression of these genes was systematically evaluated in the previously collected 62 CTCL patient biopsy samples using RT-PCR. The results of RT-PCR analysis are documented in Fig. 1 and Supplementary Figs. S1 to S3.

RT-PCR gene expression analysis confirms our previous microarray data and indicates that in CTCL there are three distinct genetic patterns/signatures (Fig. 1). Specifically, poor prognosis cluster 1 genes were significantly upregulated in cluster 1 patients and, to our surprise, were also heterogeneously expressed in cluster 3, but not in cluster 2 patients (Fig. 1A; Supplementary Fig. S1). At the same time, intermediate prognosis cluster 3 genes were selectively upregulated in cluster 3 patients with a modest overlap with cluster 1, but no overlap with cluster 2 patients (Fig. 1C; Supplementary Fig. S3). Favorable prognosis cluster 2 genes were heterogeneously upregulated in cluster 2 patients with some degree of coexpression in cluster 3 patients (Fig. 1B; Supplementary Fig. S2). These findings indicate that, biologically, there is a significant overlap between cluster 1 and cluster 3 as well as between cluster 2 and cluster 3 patients, but not between clusters 1 and 2. In this model, cluster 3 may represent an intermediate disease form between clusters 1 and 2, being located on this putative spectrum closer to cluster 1 than to cluster 2 (Fig. 1).

Correlation of molecular clustering in CTCL with clinical disease outcomes. In the previous study, we correlated the microarray gene expression with the prospective 3-year clinical follow-up from the time of biopsy (10). At that time, the clinical follow-up results did not reach statistical

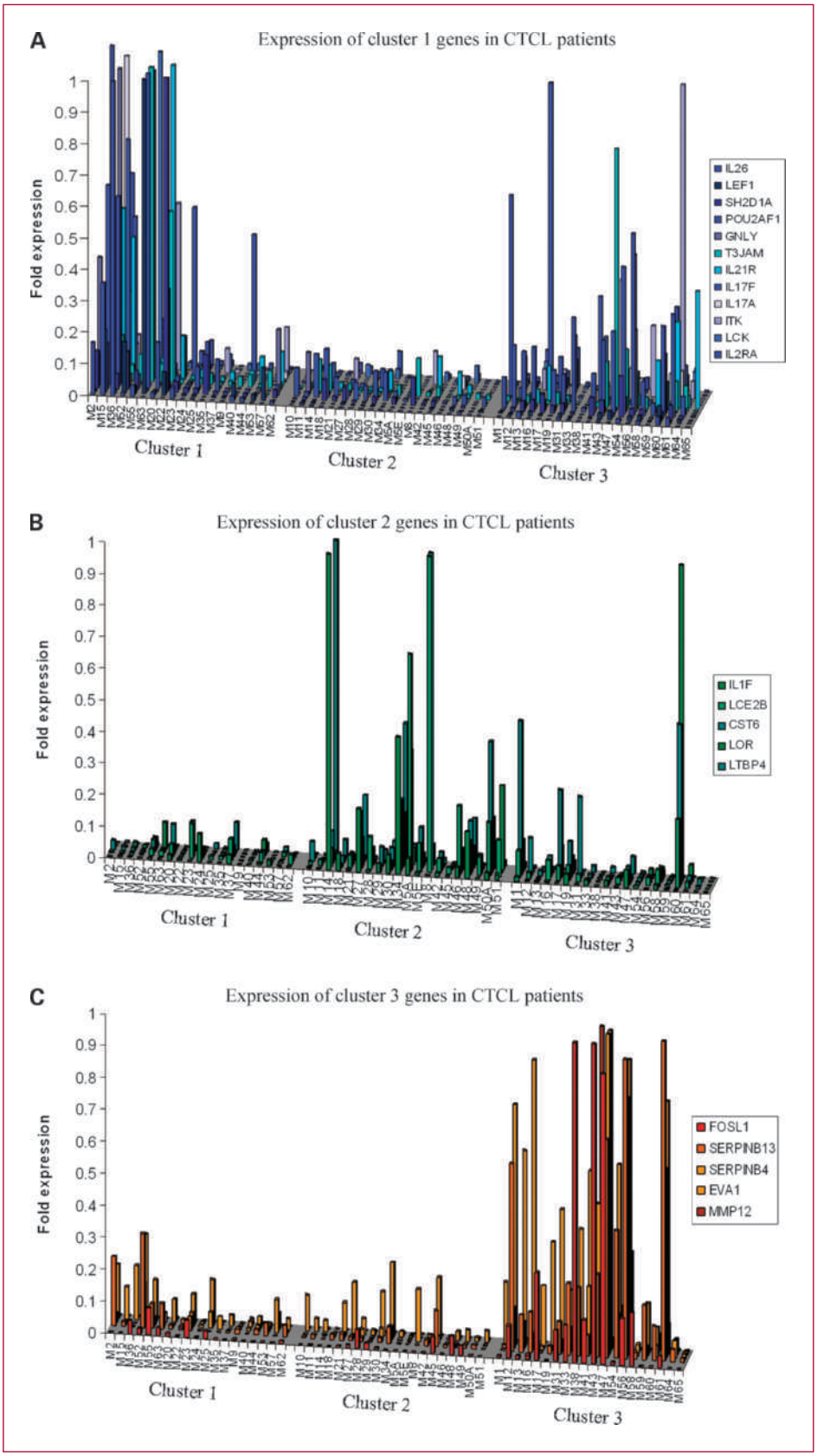

Fig. 1. Validation of previous microarray results: RT-PCR gene expression analysis in 62 CTCL patients. Quantitative RT-PCR expression of selected genes that were documented to be upregulated based on the previous microarray analysis in cluster 1 (A), cluster 2 (B), and cluster 3 (C) patients (10).

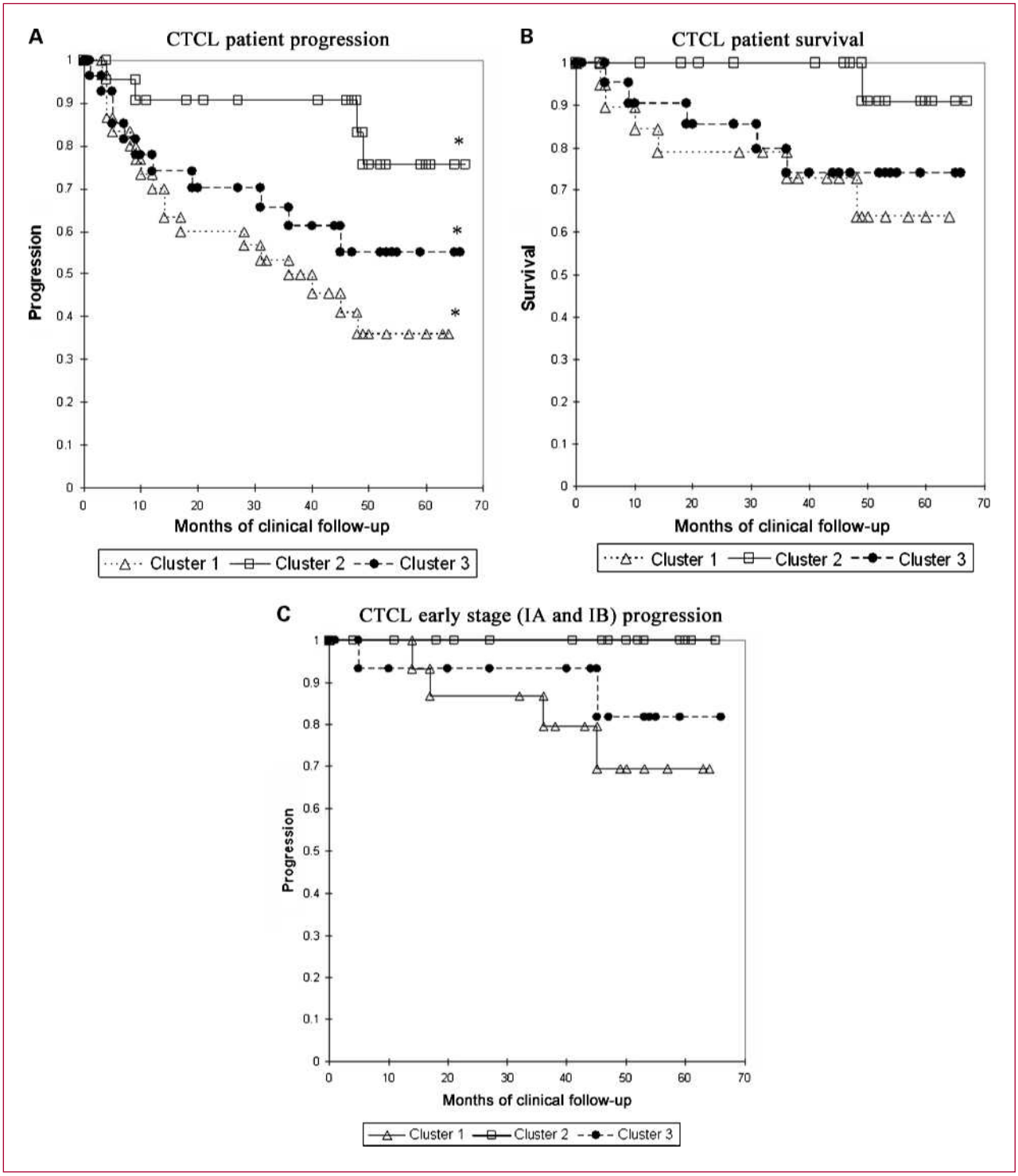

Fig. 2. Kaplan-Meier analysis of CTCL patients. A, CTCL disease progression ($P = 0.019$). B, CTCL survival ($P = 0.136$). C, progression of patients with stage I disease ($P = 0.107$).

significance. Whereas 36 months is a significant period of time, previous clinical experimental work documents that in CTCL patients that are "destined" to progress, the progression may take up to 5 years to occur from the time of diagnosis (9). Also, it is notable that only rare patients will experience progression after 5 years of stable disease (9). Thus, we wanted to extend the time of clinical follow-up to 6 years (2003-2009) and correlate the new microarray/

RT-PCR genetic data with the 6 years or 72 months of prospective clinical analysis from the time of biopsy. The Kaplan-Meier analysis of this clinical follow-up is presented in Fig. 2.

The new extended 6-year clinical analysis of CTCL progression confirms our previous observations and shows that cluster 2 has the least, cluster 1 has the most, whereas cluster 3 has an intermediate number of progression events (i.e., advancement to a higher CTCL stage and/or death; Fig. 2A). Log-rank test of the presented Kaplan-Meier analysis documents that these three clusters are statistically different ($P = 0.019$). The highest statistical difference is observed between the worst and the best prognosis clusters 1 and 2, respectively ($P = 0.004$).

Similarly, with respect to survival, cluster 2 patients enjoy a favorable 6-year survival of 91%, cluster 1 has a poor survival of only 64%, whereas cluster 3 has an intermediate survival of 74% (Fig. 2B). A statistically significant

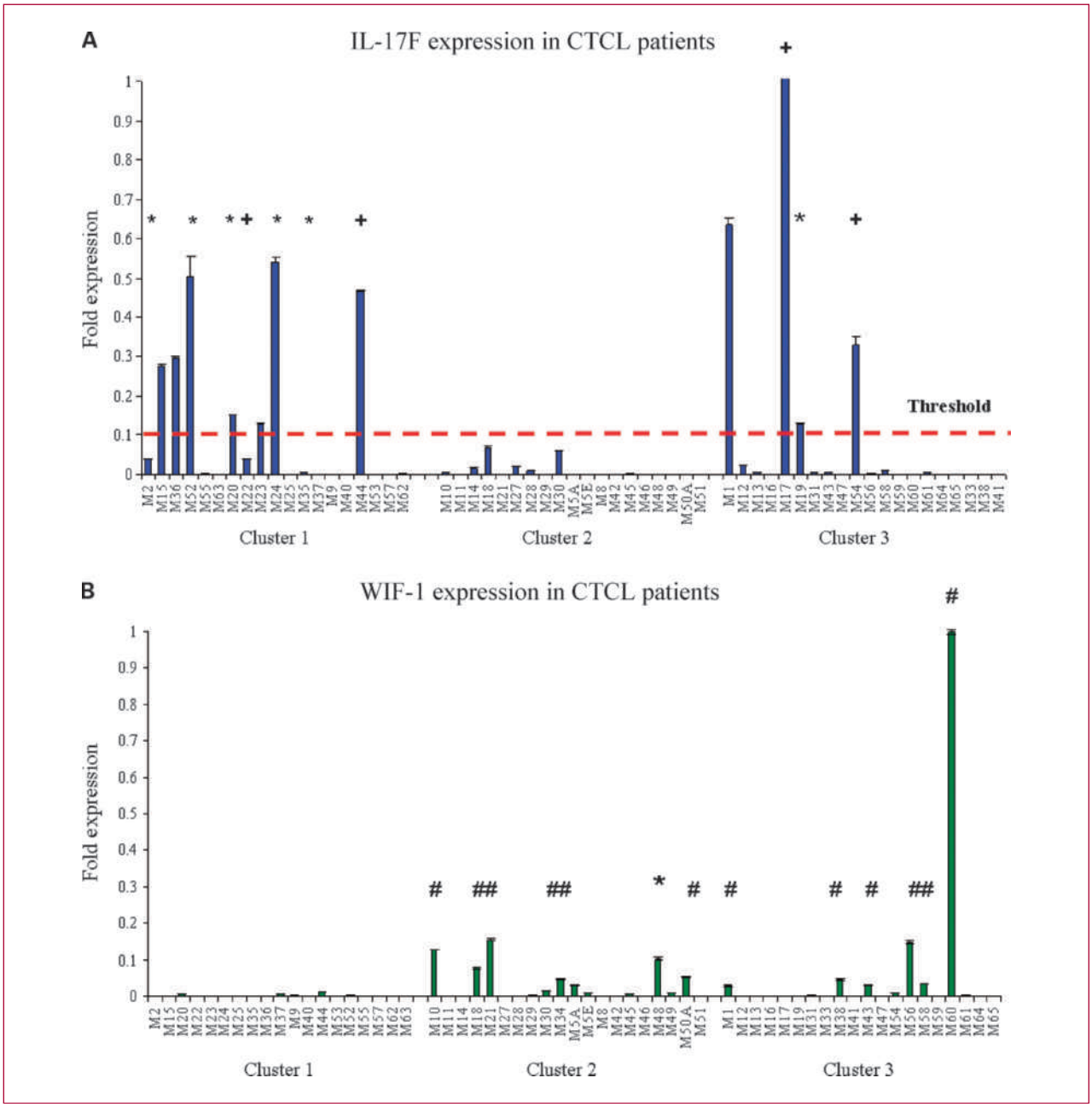

Fig. 3. Expression of molecular markers in cluster 1 to 3 CTCL patients. A, the IL-17F marker is expressed in cluster 1 and 3 patients and is upregulated in a number of patients with progressive disease (*, progression; +, progression and death). B, WIF-1 is expressed in cluster 1 and 3 favorable prognosis patients (#, stage I stable disease; *, progression). Patient M48 was diagnosed in 1998 with advanced disease, progressed to stage III in 2003 and to stage IV in 2004, and is currently doing well on appropriate therapy.

survival difference was observed between clusters 1 and 2 ($P = 0.035$). However, due to a low number of events, the survival differences between the three clusters did not reach statistical significance ($P = 0.136$).

Because each cluster had a large number of stage I disease patients (Table 2), we specifically analyzed the progression of these patients toward more advanced disease (i.e., stage IIB and beyond) with respect to their genetic clusters. According to the analysis presented in Fig. 2C, cluster 1 stage I patients had the highest 6-year progression rate of 31%, whereas cluster 3 stage I patients had an intermediate progression rate of 19%. Strikingly, none of the cluster 2 stage I patients had progressed toward advanced disease (i.e., progression rate of 0%) in the period of 6 years. It is also notable that cluster 2 had the largest number of stage I patients (i.e., 18 of 20 stage I patients in cluster II versus 11 of 20 patients in cluster 1 and 14 of 22 patients in cluster 3). A statistically significant difference was observed in the comparison of cluster 1 and cluster 2 early-stage CTCL patients ($P = 0.035$) with regard to progression. However, the three-cluster analysis for early CTCL stage progression did not reach statistical significance ($P = 0.107$). In addition, cluster 2 had only one case of stage III disease patient (patient M48). This patient was diagnosed in 1998 and progressed to stage III in 2003, at which point a biopsy was taken for the study. This patient progressed to stage IV disease in 2004, and despite the poor prognosis typically associated with this stage, he continued to maintain good health on appropriate therapy and regularly came for his clinical follow-up at the Brigham and Women's Hospital. These data suggest that stage I and possibly advanced-disease patients, who belong to molecular cluster 2, have a favorable prognosis in comparison with CTCL patients in other clusters.

Molecular markers for poor versus favorable disease prognosis. The initial microarray analysis of gene expression documented that cluster 1 patients upregulated a number of signaling pathways involved in immune response and T-cell activation, including interleukin (IL)-2 receptor signaling, the tumor necrosis factor pathway, the Th17 pathway, and others (10). Current RT-PCR analysis successfully confirmed the upregulation of these genes and pathways in cluster 1 patients (Fig. 1A; Supplementary Fig. S1). We further wanted to question whether any of these genes/pathways are predictive for progression. We performed a RT-PCR analysis that specifically highlighted Th17 pathway genes (i.e., IL-17A, IL-17F, IL-26, and IL-21 receptor) that were preferentially upregulated in cluster 1 patients (Figs. 1A and 2A; Supplementary Fig. S4). Furthermore, the expression of IL-17F was observed only in cluster 1 and 3, but not cluster 2, patients and seemed to strongly correlate with an advanced and/or progressive disease (Fig. 3A).

On the other end of the spectrum, microarray analysis for cluster 2 patients documented upregulation of a number of structural genes and pathways involved in keratinocyte and epidermal differentiation and proliferation, including cystatin, involucrin, loricrin, filaggrin, late cornified envelope (LCE2B), WNT, and transforming growth factor-β signaling (10). Furthermore, in contrast to clusters 1 and 3, cluster 2 showed minimal upregulation of immune response genes. Upregulation of the aforementioned structural and signaling genes in cluster 2 patients is documented in Figs. 1B and 3B and Supplementary Fig. S2. Notably, for WIF-1, a member of WNT signaling pathway, the expression is restricted to cluster 2 and 3 patients and seems to correlate with a favorable disease outcome for stage I patients (Fig. 3B). Furthermore, WIF-1 was also upregulated in the case of M48 stage III disease patient, who performed significantly better than expected for his disease stage (Fig. 3B). Thus, the expressions of proliferation, apoptosis, inflammatory response, and especially Th17 genes seem to be predictive of progressive and recalcitrant disease in stage I and advanced CTCL patients, whereas the expression of WIF-1 and structural genes and the lack of expression of inflammatory response genes seem to correlate with an indolent and favorable disease course.

Discussion

In this study, we have confirmed the expression levels of a select group of genes in previously identified CTCL clusters by RT-PCR. This RT-PCR analysis revealed a significant degree of genetic overlap between clusters 1 and 3 and between clusters 2 and 3, but no such overlap could be observed between clusters 1 and 2 (Fig. 1). Thus, cluster 3 seems to be a biologically distinct cluster that also serves as an intermediate link between clusters 1 and 2. Based on the 6 years of clinical analysis, the identified clusters associated with statistically significant differences in clinical outcomes, where, with respect to progression, cluster 1 had the worst, cluster 2 had the best whereas cluster 3 had an intermediate prognosis (Fig. 2A, $P = 0.019$). With respect to survival, cluster 1 had a significantly poor prognosis of 64% in comparison with 91% survival in cluster 2 ($P = 0.037$), whereas the intermediary cluster 3 had a survival rate of 74% (Fig. 2B). With respect to the stage I disease patients, clusters 1 and 3 had a significantly worse prognosis than cluster 2, where no stage I patients progressed toward advanced disease during the 6 years in the study (Fig. 2C). Comparison of cluster 1 and cluster 2 early-stage CTCL patients revealed a statistically significant difference in disease progression ($P = 0.035$). Finally, select analysis of gene expression suggests that Th17 pathway genes (e.g., *IL-17F*) are expressed in cluster 1 and cluster 3 advanced-disease patients and in early-stage patients that are likely to progress to advanced disease, whereas WNT signaling genes (e.g., *WIF-1*) are upregulated in indolent stage I and advanced-disease patients with favorable prognosis (Fig. 3).

Whereas the etiology of CTCL remains largely unknown, the current work suggests that progressive disease is associated with ongoing expression of immune response, viral response, and proliferation/apoptosis genes by the infiltrating neoplastic T cells (10). This notion is further supported

by other studies that, similar to our work, document upregulation of STAT1, STAT3, STAT5, tyrosine kinases, tumor necrosis factor signaling genes, proliferation/apoptosis genes, and other immune response genes in progressive CTCL (10, 18–20). It is further notable that a number of recent reports indicate that Th17 cells may be contributing toward carcinogenesis of CTCL (21, 22).

Based on these findings, a reasonable molecular mechanism may be postulated, where neoplastic T cells that elaborate an extensive array of inflammatory cytokines and promote local activation of immune response mediator cells and keratinocytes are likely to accelerate proliferation and generate higher levels of oxygen radicals and other toxic metabolites. Such microenvironment coupled with increased proliferation and apoptosis may further disrupt tissue homeostasis, promote genomic instability, and lead to CTCL progression. In this scenario, upregulated cytokines and immune response genes act as accelerators of cancer progression. On the other hand, in the disease where neoplastic T cells are not hardwired to elaborate these inflammatory cytokines, there is less drive toward genomic instability and cancer progression and keratinocytes are able to maintain tissue homeostasis (as evidenced by upregulation of the differentiation/structural component genes). Thus, without such acceleration, the disease seems to follow a more indolent course.

In the most advanced Sezary cell stage of CTCL, much experimental work has been accomplished to identify a specific genetic signature pattern for these cells in peripheral blood mononuclear cells. According to the recent work by Nebozshyn et al. (23), expression of *STAT4*, *GATA-3*, *PLS3*, *CD1D*, and *TRAIL* genes is able to identify Sezary syndrome cells with 90% accuracy, whereas a more expanded 10-gene expression analysis is able to predict Sezary syndrome patients that are likely to succumb to their disease in less than 6 months (24). Such research progress is encouraging because it suggests that, with further work, we may soon be able to offer personalized diagnosis and therapy to CTCL patients with early as well as advanced disease stages.

In conclusion, the current work documents three distinct CTCL disease clusters and indicates that it may be possible to use a select group of molecular markers to assign CTCL patients to a specific cluster. Early identification of poor versus favorable prognosis patients will enable us to offer more aggressive and timely treatment options for these patients while sparing patients with an indolent disease from exposure to toxic medications. These data provide valuable clinical and biological insights into CTCL disease etiology and clinical behavior. Although the presented data are intriguing, these results will need to be validated by a larger genetic analysis of CTCL patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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