Original Research

Gene expression profiling and immune cell-type deconvolution highlight robust
disease progression and survival markers in multiple cohorts of CTCL patients

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Abstract

CTCL follows different courses depending on the clinical stage at the time of diagnosis. Patients with early stage Mycosis Fungoides (MF) variant of CTCL may experience an indolent course over decades, whereas patients with advanced MF and Sézary Syndrome (SS) disease at diagnosis, often succumb within 5 years. Even within early stage CTCL/MF, a minority of patients will progress to more advanced stages. We recently generated RNA sequencing data on 284 CTCL-relevant genes for 157 patients and identified differentially expressed genes across stages I-IV. In this study, we aim to validate robust molecular markers linked to disease progression and survival. We performed multiple hypothesis testing-corrected analysis of variance (ANOVA) on the expression of individual genes across all CTCL samples and early stage (<IIA) CTCL/MF patients. We used in silico immune cell-type deconvolution from gene expression data to estimate immune cell populations. Based on the analysis of all CTCL samples, we identified TOX, FYB, and CDS2 as predictors of disease progression and poor survival. Among early stage (<IIA) CTCL/MF patients, these 3 genes, along with CCR4, were valuable to predict disease progression. We validated these 4 genes in 3 independent, external Sézary Syndrome patient cohorts with RNA-sequencing data. In silico immune cell-type deconvolution revealed that neutrophil infiltration in early stage MF conveyed a higher risk for disease progression. Also, NK cell infiltration in late stage MF/SS correlated with improved survival. TOX, FYB, CCR4 and CDS2 are robust disease progression and decreased survival biomarkers in CTCL.

Introduction

Cutaneous T-Cell Lymphomas (CTCL) display a characteristic malignant clonal T lymphocyte skin infiltrate. They remain uncommon (about 10 to 11 cases per million person-years in the United States and Canada). Most patients present with skin-limited CTCL that follows a smoldering course and does not progress to affect the blood or visceral organs, but approximately 15 to 20% will progress to a potentially lethal disease with multi-organ involvement and survival of less than 5 years. Cutaneous lymphomas have a wide range of clinical presentations, including mycosis fungoides (MF) and the hematogenous/leukemic form, Sézary Syndrome (SS). In addition to polymorphic clinical variants, even classic CTCL/MF can mimic benign, more common inflammatory conditions such as psoriasis, contact or drug induced spongiotic dermatitis, lichen planus and psoriasis, contributing to as much as 6-year delay in diagnosis from the initial presentation. Predicting disease progression and overall survival in CTCL are challenging endeavors, but could help stratify early stage CTCL patients as those with indolent course and those at a higher risk for progression towards advanced stages. Clinical disease stage at the time of diagnosis directly influences prognosis, as expected. Search for molecular biomarkers has yielded clues into disease etiology and progression. Gene expression profiling studies have identified an association between adverse prognosis and high expression of TOX, GTSF1, NOTCH1, CCR4, ITK, FYB, SYC1, LCK or miR155, miR21, and let-7i differentially-expressed genes (DEGs). Recently, a miRNA classifier, based on a linear combination of 3 miRNAs (miR-106b-5p, miR-148a-3p, and miR-338-3p), successfully separated early stage CTCL patients into two groups (high risk and low risk) with different outcomes based on disease progression and survival. From gene expression data, computational methods to enumerate individual cell types, a
process called RNA deconvolution, can also reveal prognostic biomarkers. One such popular deconvolution algorithm, based on support vector regression and thus less sensitive to outliers, is CIBERSORT.

Recently we obtained targeted RNA-sequencing-based gene expression data from 157 patients with either CTCL or benign skin dermatoses (including skin tags, eczema, psoriasis among others), mostly from formalin-fixed paraffin-embedded (FFPE) clinical samples. Our primary analysis validated previously identified DEGs and confirmed their ability to distinguish benign from CTCL samples. We also performed subgroup analysis to identify additional DEGs and explored biases arising from processing FFPE samples on the TruSeq targeted RNA sequencing platform.

In this study, we use our TruSeq gene expression data from our previously characterized CTCL patient cohort to identify robust disease progression and survival markers, followed by external validation in publicly-available sequencing-based datasets generated from Sézary Syndrome patients. We also perform RNA deconvolution to identify immune cell types with possible clinical implications.

Results

High expression of TOX, FYB, and CD52 in CTCL samples correlates with greater risk of disease progression and poor survival

To robustly identify genes for which differential expression is strongly associated with disease progression, we performed one-way ANOVA across 134 Formalin Fixed and Paraffin Embedder (FFPE) samples of lesional CTCL skin from 110 patients with available clinical data from our cohort (Supplementary Table 1 for clinical characteristics of CTCL patients). After multiple hypothesis correction using Benjamin-Hochberg method, high expression of TOX (corrected \( p = 0.005 \)), FYB (\( p = 0.005 \)) and CD52 (\( p = 0.015 \)) were associated with CTCL disease progression (Fig. 1A-C). Survival analyses for these 3 genes confirmed their role in CTCL prognosis, as significantly-decreased disease-specific survival was observed for high expression of TOX (\( p = 0.020 \); log-rank test), FYB (\( p = 0.004 \)), and CD52 (\( p = 0.032 \)) (Fig. 2A-C). Hazard ratios adjusted for age (along with 95% confidence intervals) were 2.03 (1.10–3.74) for TOX, 2.44 (1.29–4.60) for FYB, and 1.94 (1.05–3.59) for CD52.

High expression of TOX, FYB, CD52, and CCR4 in early stage CTCL samples is associated with disease progression

Identification of molecular biomarkers involved in discrimination between early stage (\( \leq IIa \)) CTCL/MF patients, who progress and the majority, who do not is a challenging task. We used one-way ANOVA analysis across a subset (64 FFPE samples of lesional CTCL/MF skin from 57 patients) of CTCL samples that corresponded to early stage disease (\( \leq IIa \)) to identify genes that could be associated with disease progression to advanced (\( \geq IIb \)) stages. Four genes were found to be significant at the \( p < 0.01 \) level: the three genes previously highlighted TOX (\( p < 0.001 \)), FYB (\( p < 0.001 \)), and CD52 (\( p = 0.008 \)), plus an additional gene, CCR4 (\( p = 0.003 \) (Fig. 3A-D). High expression of these four genes heralds disease progression in early stage CTCL/MF.

While stage IA patients have normal life expectancy, those with stage IB experience a mild decrease in survival. Analyzing a subset of these patients, specifically those with stage IB disease who either progressed to stage IIB (8 patients) or remained in stage IB (15 patients), higher expression of the four aforementioned biomarkers was observed in samples from patients who progressed from stage IB to IIB compared to those who did not (TOX mean TPM 3122 vs. 1578; CCR4 mean TPM 7570 vs. 5074; FYB mean TPM 9574 vs. 4503; CD52 mean TPM 103,543 vs. 73,074).

Higher RNA-sequencing expression of TOX, FYB, CD52 and CCR4 in Sézary patients compared to controls from external cohorts

To validate our expression based-biomarkers, we searched PubMed and NCBI Gene Expression Omnibus (GEO), NBI Short Read Archive (SRA) and EMBL-EBI European Genome-Phenome Archive (EGA) for RNA sequencing-based datasets pertaining to CTCL/MF/SS. We obtained RNA-Seq data from three studies focusing on Sézary Syndrome patients, encompassing 14 affected patients and 3 healthy controls for Ungewickell et al. and Choi et al. combined, and 34 affected patients and 5 healthy controls for Wang et al. All samples analyzed in these three studies were from peripheral blood. Using non-parametric resampling to enable scale-free comparisons between different study designs and to decrease batch effect, gene expression levels were compared for TOX, FYB, CD52 and CCR4 between affected (i.e., CTCL) and non-affected individuals. All four genes were significantly enriched amongst Sézary syndrome patients in Ungewickell et al. and Choi et al.: TOX (\( p < 10^{-5} \)), FYB (\( p = 0.004 \)), CD52 (\( p < 10^{-5} \)), and CCR4 (\( p < 10^{-5} \)) (Table 1A). For Wang et al., more samples had TPM = 0 for the genes of interest, affecting results given the non-parametric approach. The ratio of mean TPM Sézary syndrome to mean TPM controls was at least \( >3 \) for all four genes (Table 1B): TOX (\( p < 10^{-5} \)), FYB (\( p = 0.0002 \)) and CD52 (\( p < 10^{-5} \)) were enriched in Sézary syndrome patients. Overall, these results indicate that our prognostication biomarkers can be applied to other cohorts as well.

In silico immune cell-type enumeration using RNA deconvolution

Leukocyte infiltration of malignant tumors, often described as the tumor micro-environment, has clinical prognostic implications. Measurement of cell heterogeneity using conventional techniques such as immunohistochemistry can suffer from artefacts or prove impractical. Gene expression signatures from pure cell lines can be used to enumerate cell composition of admixed, heterogeneous samples using RNA deconvolution. RNA deconvolution can be used by itself as a novel biomarker. Malignant inflammation from the tumor micro-environment influences CTCL tumorigenesis. We first used CIBERSORT, a robust algorithm, based on support vector regression, to determine the immune
Figure 1. Gene expression presented in Transcripts per million (TPM) according to disease progression status for TOX (A), FYB (B), and CD52 (C) in all CTCL samples. Quartile boxplots are shown.
Figure 2. Survival analysis plots of all CTCL samples according to gene expression for TOX (A), FYB (B), and CD52 (C). Samples are dichotomized based on gene expression levels in TPM after finding an optimal cut-off; samples with TPM below cut-off labelled as low (solid line) and samples with TPM above cut-off labelled as high (dotted line). Survival plots correspond to Cox proportional hazards regression models.
composition of our 134 CTCL FFPE samples obtained from 110 patients and then tested whether there was an association with disease progression in early stage (i.e. stage ≤IIA) CTCL/MF and survival advantage in late stage (≥IIB) CTCL (MF and SS).

In early stage CTCL, tumors from patients who progressed show a higher degree of neutrophilic infiltration (ANOVA; p = 0.032; Fig. 4A). Previous reports have indicated a link between high expression of IL-17A and IL-17F, neutrophil infiltration of CTCL tumors and increased risk of CTCL progression.36,37 Unfortunately, IL-17 genes were not included in our TruSeq panel. We observed that high levels of IL-17A and IL-17F, but also IL-17C, IL-17D and IL-17E mRNA, were found amongst Sézary patients (p < 10^{-5}) along with a lower expression of IL-17B (p < 10^{-5}) (Table 2A) for the two pooled cohorts of Ungewickell et al. and Choi et al. For the Wang et al. Sézary cohort, more samples had IL-17 genes with TPM = 0. The ratio of mean TPM Sézary to mean TPM controls was at least >1.5 for IL-17B, IL-17D and IL-17E expression (Table 2B); IL-17A (p < 10^{-5}), IL-17B (p < 10^{-5}) and IL-17F (p < 10^{-5}) were enriched in Sézary patients.

In late stage CTCL (stage IV), patient samples lacking NK T lymphocyte infiltration were associated with a faster demise (50% survival about 0.5 years if NK T cell <5% vs. more or less 1.5 years if NK T cell >5%, as obtained by CIBERSORT) (Log-rank test; p = 0.032; Fig. 4B). This is consistent with reactive NK T lymphocytes as cytotoxic effectors to control CTCL progression, an infiltration linked with a better prognosis.38

For immune cell type enumeration, we did not observe any significant association between 2 other cell types (CD8+ T lymphocytes or regulatory T lymphocytes) and disease progression/survival.

### Discussion

We previously reported TOX, FYB, GTSF1, and CCR4 differentially-expressed genes (DEGs) when comparing CTCL and benign dermatoses, conditions that mimics this cancer.24 In this study, we confirmed their importance to clinical prognostication by showing that TOX and FYB are associated with early stage disease progression. Also, in all CTCL samples across stage I-IV disease these genes were associated with disease progression/survival.

| Genes | Ratio mean TPM | Fraction Genes with TPM > GOI – SS | Fraction Genes with TPM > GOI – Control | Corrected p-value |
|-------|----------------|-----------------------------------|----------------------------------------|------------------|
| TOX   | 5.38           | 0.1896                            | 0.1123                                 | < 10^{-5}        |
| FYB   | 1.90           | 0.0429                            | 0.0288                                 | 0.004            |
| CD52  | 1.99           | 0.0017                            | 0.0007                                 | < 10^{-5}        |
| CCR4  | 3.56           | 0.0735                            | 0.0231                                 | < 10^{-5}        |

TPM = Transcripts per Million, GOI = Gene-Of-Interest (Tested gene), SS = Sézary Syndrome.
progression and poor disease-related survival. In other cohorts, high expression of TOX, a protein implicated in T-cell development through chromatin regulation, is known to lead to CTCL tumor progression and poor overall prognosis, where as a minimal level of TOX mRNA expression can be observed in normal skin or benign dermatoses. When over-expressed, FYB, a T cell adapter protein involved in T cell activation, has also been associated with advanced disease in other cohorts.

In our cohort, CCR4 was found to be significantly associated with disease progression in early stage CTCL only. CCR4, a chemokine involved in T cell homing to the skin, is expressed in approximately 40% of CTCL patients, and correlates well with skin infiltration by these cells. High expression of CCR4 mRNA has been linked to disease progression in other cohorts. Importantly, CCR4 is an actionable target. Moga-nulizumab, a humanized anti-CCR4 monoclonal antibody, showed overall response rates (complete and partial responses) of 28.6% in MF patients, and 47.1% in Sézary patients, during a phase I/II clinical trial. It can significantly reduce levels of CCR4-expressing malignant T cells.

We also found that high expression of CD52 leads to disease progression in early stage CTCL/MF, and to disease progression and decreased survival in all CTCL patients, a novel finding. CD52 is a glycosylphosphatidylinositol-anchored negatively-charged glycoprotein with potential roles in T cell migration and co-stimulation of the immune response. CD52 is highly expressed in both SS and MF compared to controls, in the latter more specifically in lesional skin. Alemtuzumab, a humanized anti-CD52 monoclonal antibody, has demonstrated efficacy in MF and SS patients with an overall response rate of 55%, but other studies in advanced stage CTCL, treatment-refractory patients showed a moderate response rate (38%), but short time to disease progression following treatment. Of note, in chronic lymphocytic leukemia, response rates to alemtuzumab were correlated with the level of CD52 expression.

Importantly, we have successfully validated our four biomarkers with RNA-Seq data in Sézary patients from other cohorts, as RNA-sequencing-based gene expression studies from skin CTCL clinical samples are sparse. Despite the fact that different tissues were analyzed in our study (skin tissue biopsy samples) and in the external validation cohort studies (peripheral blood samples), biomarkers were reproducibly enriched in skin CTCL samples and in blood Sézary samples, strongly supporting their involvement in tumorigenesis in both conditions.

RNA deconvolution from gene expression data also helped us identify clinically-relevant biomarkers. Our in silico immune cell-type enumeration using RNA deconvolution with the CIBERSORT algorithm yielded two cellular biomarkers related to CTCL tumorigenesis. First, advanced stage CTCL samples with the least (<5%) NK T lymphocyte infiltration showed decreased survival times. NK T lymphocytes play a role in approximately 40% of CTCL patients, and correlates well with skin infiltration by these cells.

Table 2B. IL-17A through IL-17F expression in the Wang et al. independent cohort of Sézary patients with RNA-Sequencing gene expression data.

| Genes | Ratio mean TPM (SS/mean TPM Control) | Fraction Genes with TPM > GOI – Control | Fraction Genes with TPM > GOI – SS | Corrected p-value |
|-------|-------------------------------------|----------------------------------------|----------------------------------|-----------------|
| IL-17A | >63.21                              | 1                                      | 0.2981                           | <10⁻⁵           |
| IL-17B | 0.89                                | 1                                      | 0.2443                           | <10⁻⁵           |
| IL-17C | 5.47                                | 0.6564                                | 0.4476                           | <10⁻⁵           |
| IL-17D | 7.95                                | 0.5131                                | 0.2735                           | <10⁻⁵           |
| IL-17E | >251.28                             | 1                                      | 0.2292                           | <10⁻⁵           |
| IL-17F | >24.65                              | 1                                      | 0.4160                           | <10⁻⁵           |

TPM = Transcripts per Million, GOI = Gene-Of-Interest (Tested gene), SS = Sézary Syndrome.

Figure 4. (A) Neutrophil immune cell fraction obtained in silico using CIBERSORT according to disease progression status in early stage (≤IIA) CTCL samples. Quartile boxplots are shown. (B) Survival analysis plots of late stage (IV) CTCL samples according to NK T lymphocyte fraction. Samples are dichotomized according to NK T lymphocyte immune cell fraction obtained in silico using CIBERSORT; samples with fraction below 0.05 labelled as low (solid line) and samples with fraction above 0.05 labelled as high (dotted line). Survival plot corresponds to Cox proportional hazards regression models.

Table 2A. IL-17A through IL-17F expression in two pooled independent cohorts of Sézary patients with RNA-Sequencing gene expression data (Ungewickell et al., Choi et al.).
in a cytotoxic response to skin malignant infiltration, resulting in better prognosis.\textsuperscript{38} An \textit{in vitro} cellular model from primary tumors demonstrated that activation of NK T cells, such as with an anti-KIR3DL2 monoclonal antibody, leads to an anti-tumoral NK cytotoxic activity.\textsuperscript{52} Second, neutrophilic infiltration of early stage CTCL lesions is linked to a progression to more advanced disease stages (>IIB) in our cohort of patients. Reports from independent cohorts are consistent with this observation in CTCL.\textsuperscript{36,37} Increased neutrophil infiltration, often calculated as a high neutrophil to lymphocyte ratio, usually heralds disease progression and decreased survival in other cancers, including melanoma.\textsuperscript{53}

Neutrophils are often recruited by cytokines produced by Th17 lymphocytes. We have found that \textit{IL-17A} and \textit{IL-17F} had increased RNA-Seq expression in Sézary patients compared to normal patients in one external cohort.\textsuperscript{27} \textit{IL-17A} and \textit{IL-17F} were both expressed preferentially in lesional skin in CTCL patients,\textsuperscript{37} but only \textit{IL-17F} has been linked to disease progression.\textsuperscript{37} A recent \textit{in vitro} study showed that increased angiogenesis might be the result of \textit{IL-17F} release.\textsuperscript{54} \textit{IL-17} expression in CTCL is likely driven by external triggers (\textit{S. aureus} bacterial toxins, UV radiation, dermatophytes, etc.) and other factors in the tumor microenvironment.\textsuperscript{55} Based on recent clinical experience, it appears that blocking \textit{IL-17} signaling does not slow CTCL progression and must be further addressed with caution. Through clinical reports\textsuperscript{56} and personal clinical experience, where CTCL patients misdiagnosed with erythrodermic psoriasis received up to 1 year of secukinumab treatment that blocks \textit{C19/20} and \textit{IL-17A} and \textit{IL-17F} had decreased \textit{CD52} expression in S. aureus patients compared to healthy skin).\textsuperscript{57} Follow-up times according to disease progression status and whether other genes would be significantly associated with disease progression status and death were established according to the diagnostic criteria of CTCL.\textsuperscript{57} We observed that blocking \textit{IL-17} signaling does not slow CTCL progression and must be further addressed with caution.

In conclusion, in this study, we confirmed the value, and cost-effectiveness, of performing TruSeq targeted RNA sequencing on clinically-obtained biopsy samples to uncover gene expression-based markers of survival and disease progression for cutaneous cancers. The biomarkers we identified robustly in our CTCL patients were validated in external cohorts, linked to CTCL tumorigenesis, and (for \textit{CCR4} and \textit{CD52}) can be targeted by monoclonal antibodies. In the future, when precision medicine becomes more routine in the clinical setting, these molecular biomarkers may play an important role in stratifying patients at higher risk of disease progression that would benefit from early systemic therapy, while avoiding unnecessary side effects from these treatment modalities in patients at low risk of disease progression and mortality.

**Methods**

**Patients and samples**

Patients originated from the University of Texas MD Anderson Cancer Center (MDACC) Research Ethics Board, which exempted us from obtaining written informed consent from patients, who earlier signed a hospital consent allowing their stored biopsy samples to be used for research. The diagnosis and clinical staging were established according to the diagnostic criteria of CTCL.\textsuperscript{57} Follow-up times according to disease progression status are shown in Supplementary Fig. 1.

**Data acquisition**

Transcripts Per Million (TPM) from previously processed TruSeq data was re-analyzed; this data has been deposited in NCBI SRA under accession number SRP114956.\textsuperscript{24,25} The following de-identified clinical data variables from the CTCL patients cohort were considered: disease progression as a dichotomous variable, and survival/vital status as a dichotomous variable. Only time from sample biopsy to either death or last follow-up were considered. If exact date was not available, then the midpoint was considered (i.e. June 2011 would become 15 June 2011). Samples were grouped as early (stage ≤IIA) vs. intermediate (stages IIB and III) vs. advanced (stage IV) CTCL, as previously described.\textsuperscript{24,25}

**Identification of disease progression and survival markers**

One-way Analysis of Variance (ANOVA) test was performed for every gene, comparing disease progression status (defined as progression to a higher clinical stage), on all CTCL samples. P-values were then corrected for multiple hypothesis testing using Benjamini-Hochberg method\textsuperscript{29} and genes with corrected P-value <0.05 were considered. Disease-specific survival analyses were performed using Cox proportional hazards regression in R (packages "survival", “KMsurv” and "OIsurv”); variables included vital status, time to death or last follow-up from sample biopsy date, age (adjustment parameter) and dichotomized gene expression based on an arbitrary TPM cut-off for each gene. For disease-specific survival analysis, p-values were computed using log-rank test.

We specifically looked at early stage MF samples, to confirm whether the same genes were found to be associated with disease progression status and whether other genes would be significant. We performed one-way ANOVA as described above. The same 3 genes, plus only one additional gene, were found using p-value <0.01 as a cut off point.

**Cell-type enumeration using RNA deconvolution**

CIBERSORT was used to perform RNA deconvolution using the standard LM22 leukocyte signature matrix, obtained from 22 immune pure cell lines\textsuperscript{23} and 100 permutations. One-way ANOVA for disease progression and survival analyses were performed for early stage MF and late stage MF/SS, respectively.

**External validation of disease progression and survival markers**

We obtained publicly-available RNA-Seq data for Sézary Syndrome patients.\textsuperscript{26-31} To prevent batch effect\textsuperscript{32} from
comparing different studies with different designs and annotations, we have converted TPM to a normalized rank, more specifically for each external case by taking the number of genes with a higher TPM value divided by the total number of reported genes in that sample. This gives rise to a non-parametric rank fraction value. We next computed p-values comparing the means of non-parametric rank fraction values between Sézary patients and control samples and, hence, was analyzed separately. Two of the three studies did not provide clinical data on patient characteristics.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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