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FISH panel for leukemic CTCL

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Keywords

cutaneous T cell lymphoma; mycosis fungoides; follicular mycosis fungoides; Sézary syndrome; fluorescence in situ hybridization; ARID1A; STAT3; STAT5B; ZEB1; FAS; CARD11; TP53; DNMT3A; MYC; RB1; ATM; CDKN2A

To the Editor

At present, no clinical tests are widely used to assess mutational burden in cutaneous T cell lymphoma (CTCL) with leukemic (L-CTCL) involvement, such as in Sézary syndrome (SS), and routine blood analysis offers limited guidance for assessing prognosis and optimizing treatment within the highly variable population of affected patients. For now, L-CTCL contrasts sharply with more common and better-characterized cancers, such as chronic lymphocytic leukemia and ductal carcinoma of the breast, for which mutation analysis stratifies prognostic subgroups and can influence disease management (Nuciforo *et al*., 2016; Van Dyke *et al*., 2016). We have developed and validated a panel of 11 fluorescence in situ hybridization (FISH) probes designed to capture gene copy number alterations (GCNAs) present in 97.5% of patients with L-CTCL as elucidated in a recent exome study (Choi *et al*., 2015). The panel includes 5 probes previously developed for TP53, MYC, RB1, CDKN2A, and ATM, as well as 6 newly designed probes for STAT3/5B, ARID1A, ZEB1, FAS, CARD11, and DNMT3A (Figure 1a). FISH technology particularly suits CTCL, a cancer whose mutational landscape is characterized by a preponderance of gene copy number amplifications and deletions compared to single nucleotide variants relatively

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Sites of experiments: New Haven, Connecticut, U.S.A.

CONFLICTS OF INTEREST:
JW, JL, and MG are listed as inventors on a provisional patent filed on several probes reported herein.

SUPPLEMENTARY MATERIAL:
Supplementary material is provided in a PDF submitted with this document.

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overrepresented in many other forms of cancer. In this study, we used the 11-probe panel to assess for genetic abnormalities in sorted or unsorted peripheral blood from 24 patients (patient characteristics in Supplementary Materials) with a range of disease presentations including SS, patch/plaque mycosis fungoides (MF), follicular mycosis fungoides (F-MF), tumor-stage mycosis fungoides, and follicular mucinosis –with evidence of blood involvement (Gibson *et al.*, 2016).

After design and production of FISH probes for STAT3/5B, ARID1A, ZEB1, FAS, and CARD11, probe hybridization sites were validated on metaphase chromosome spreads. Written informed consent of 24 patients who had a confirmed or suspected diagnosis of CTCL was obtained in accordance with protocols approved by the Institutional Review Board of Yale School of Medicine. Populations enriched for abnormal CD3+CD4+ lymphocytes, most frequently also CD26− and/or CD7−, were purified from Ficoll-isolated PBMC by either flow cytometric sorting or by magnetic bead isolation. Samples underwent fixation and overnight FISH hybridization with the panel of 11 probes, and probe signals were quantified by fluorescence light microscopy at 100 or 200 nuclei per probe (Figure 1b). CD8+ T cells collected as control populations were used to generate cutoffs (Supplementary Materials) for positive FISH abnormalities at the 99th percentile of binomial distribution of proportions calculated for each probe (Wiktor *et al.*, 2006).

GCNAs were detected by FISH (Figure 2a) in 10 of 10 patients meeting International Society of Cutaneous Lymphoma (ISCL) criteria for SS and B2 stage blood involvement, 1 patient with F-MF not meeting B2 criteria, 1 patient with tumor-stage MF meeting B2 criteria, and 2 patients with MF one of whom met B2 criteria. No abnormalities were detected by FISH in the other 10 patients, of whom only 1 patient (with MF) met B2 involvement by one criterion (41.5% CD7− of CD4+ T cells; sorted CD7− cells). Among the 9 remaining patients with no detected GCNAs, 6 had a diagnosis of MF; 1 had a diagnosis of F-MF; 1 had a diagnosis of follicular mucinosis in association with Crohn's disease (elevated CD4/8 ratio of 5.7, negative T cell receptor (TCR) PCR analysis for clonality); and 1 had atypical angioedematous plaques with initially positive (but subsequently negative) TCR clonality by PCR. Overall, of patients meeting revised 2007 ISCL criteria for B2 blood involvement, 12 of 13 patients (92%) had GCNAs detected by FISH.

Proportions of FISH-identified GCNAs present per gene in patients with Sézary syndrome did not significantly differ from those observed in a recent large-scale exome study on a separate patient cohort (p-values 0.27–1.00, Fisher's exact test, Figure 2b). One patient had an unanticipated single deletion of CARD11 in 92% of cells scored. CARD11 promotes the T cell receptor-mediated activation cascade and has been found amplified in CTCL exome data (Choi *et al.*, 2015). However separate comparative genomic hybridization (CGH) data have shown a more balanced frequency of amplification and deletion in CARD11 (Kiel *et al.*, 2015), and its functional significance in CTCL cells remains incompletely explored. Multiple atypical amplifications, including 4x amplifications in ARID1A, DNMT3A, RB1, and FAS, were seen in a separate patient with longstanding disease.

Much research remains to be completed relating patient outcomes and treatment susceptibilities to the status of driver genes in CTCL. Within our cohort of patients, specific

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GCNAs of note include 2 of 10 SS patients with double amplifications of the well-characterized oncogene MYC (Dang, 2012) found within broad amplifications on chromosome 8q in CTCL; joint amplification of several genes has complicated the interpretation of the significance of individual components in this region (Choi *et al.*, 2015). Recent progress on investigational anti-Myc therapeutics (Stellas *et al.*, 2014) may offer a targeted approach to assess the role of this oncogene. Also notable was 1 SS patient with homozygous deletion of ZEB1 and peripheral blood T cell count above 20,000/μL by 8 months after initial clinical presentation with erythroderma. ZEB1, a transcriptional repressor of IL-2 (Wang *et al.*, 2009) and contributor to TGF-β1 mediated growth inhibition in adult T cell leukemia/lymphoma (Nakahata *et al.*, 2010), has been found homozygously deleted in 10% of exome-sequenced L-CTCL patient samples; homozygous deletion in a mouse model has given rise to fatal T cell lymphomas in 84% of affected animals (Hidaka *et al.*, 2008). Yet the relative response of CTCL harboring these and other mutations to immune-modulating therapies, biologic entities, or traditional chemotherapy is currently unknown.

We believe that the presented 11-probe FISH panel may offer the capacity to facilitate the diagnosis of L-CTCL, while also providing genetic status based on many of the most commonly represented GCNAs reported in CTCL. Since many of the genes represented have only recently been published as oncologic drivers of CTCL, we are currently unable to correlate these GCNAs with clinical outcomes; however, as outcomes data from genetic studies accumulate in the near future, this panel may also provide a helpful tool for prognosis and treatment stratification with advantages including rapid turnaround and ease of clinical implementation in hospitals performing FISH studies. We suggest the use of this 11-probe FISH panel to enhance the efficient testing of patients with L-CTCL to the standards of many other cancers of the blood, and suggest the potential utilization of FISH in personalized medicine for CTCL patients. Applying the panel to an expanded patient cohort will be necessary before the full utility, sensitivity, and specificity of FISH analysis in CTCL diagnostics can be conclusively determined. For investigative purposes, correlating GCNAs with expression levels and other biomarkers may reveal critical pathways underpinning disease behavior and new targets for therapeutic intervention. For clinical use, a practical and cost-effective strategy to consider would be to utilize a four probe subset consisting of the two most informative probes assessing deletion (TP53, ARID1A) and amplification (MYC, STAT3/5B), consistent with a previously published study (Vermeer *et al.*, 2008). Combining exome data (Choi *et al.*, 2015) with our patient cohort, GCNAs have been found present in this subset of genes in 96.2% of CTCL patients with stage B2 blood involvement (two-sided 95% Clopper Pearson confidence interval 87.0%–98.8%).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations in this paper

CTCL	cutaneous T cell lymphoma
L-CTCL	leukemic cutaneous T cell lymphoma
MF	mycosis fungoides
F-MF	follicular mycosis fungoides
MF-T	mycosis fungoides with tumor
SS	Sézary syndrome
FISH	fluorescence in situ hybridization
GCNA	gene copy number alteration
CGH	comparative genomic hybridization
PBMC	peripheral blood mononuclear cells
ISCL	International Society of Cutaneous Lymphoma
IL-2	interleukin-2

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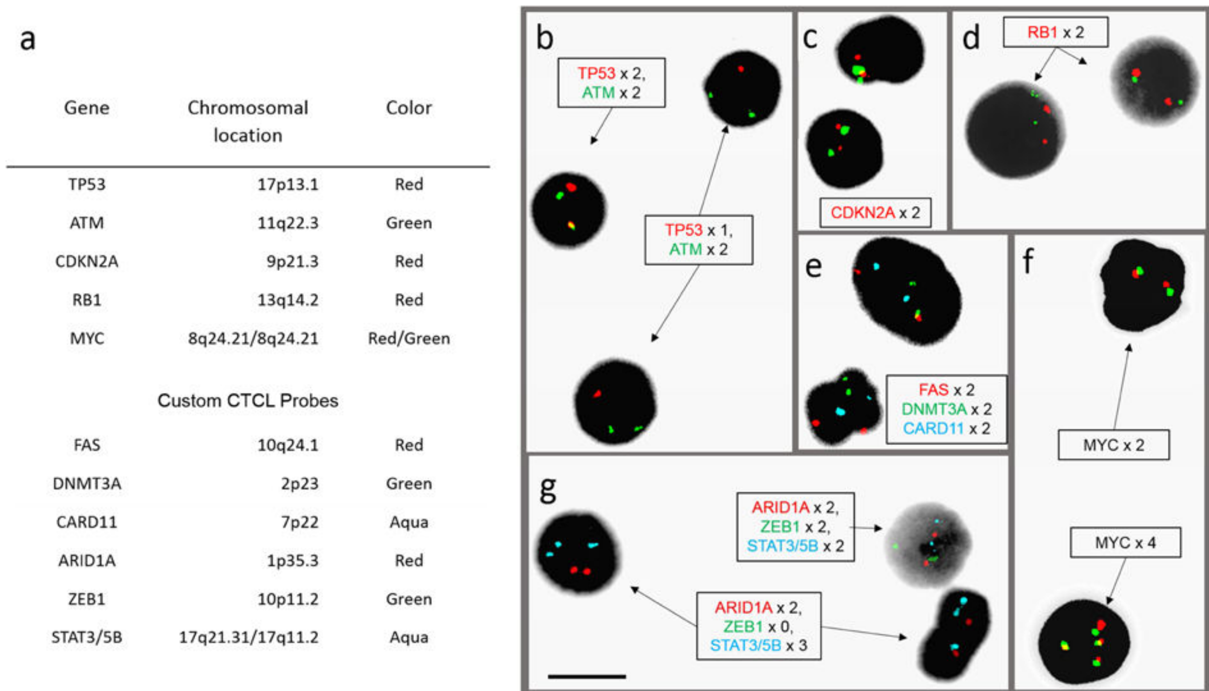

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Gene	Chromosomal location	Color
TP53	17p13.1	Red
ATM	11q22.3	Green
CDKN2A	9p21.3	Red
RB1	13q14.2	Red
MYC	8q24.21/8q24.21	Red/Green
Custom CTCL Probes		
FAS	10q24.1	Red
DNMT3A	2p23	Green
CARD11	7p22	Aqua
ARID1A	1p35.3	Red
ZEB1	10p11.2	Green
STAT3/5B	17q21.31/17q11.2	Aqua

Figure 1. L-CTCL FISH panel composition and probe microscopy

(**a**) Table of genes, chromosomal locations, and fluorescent colors for 11 clinically validated probes used in the CTCL FISH panel. (**b–g**) Post-hybridization microscopy images using 11-probe FISH panel on peripheral blood samples from patients with L-CTCL and abnormal FISH results. (**b**) Field with cell showing normal copy number of two TP53 (red) signals and two ATM (green) signals, and two cells with deletion of one copy of TP53. (**c**) Cells with two copies of CDKN2A (red) and 9q12 control probe (green). (**d**) Two cells with two copies of RB1 (red) and 13q control (green). (**e**) Signals for DMNT3A (green), CARD11 (aqua), and FAS (red), both cells showing two copies of each. (**f**) Cell on top with two copies of MYC stained with the red and green probes (both for MYC); cell below showing four copies of MYC. (**g**) Signals for ARID1A (red), ZEB1 (green), and STAT3/5B (aqua); one cell with normal copy numbers and two cells with homozygous deletion of ZEB1 and amplification of STAT3/5B. Black bar = 10 µm.

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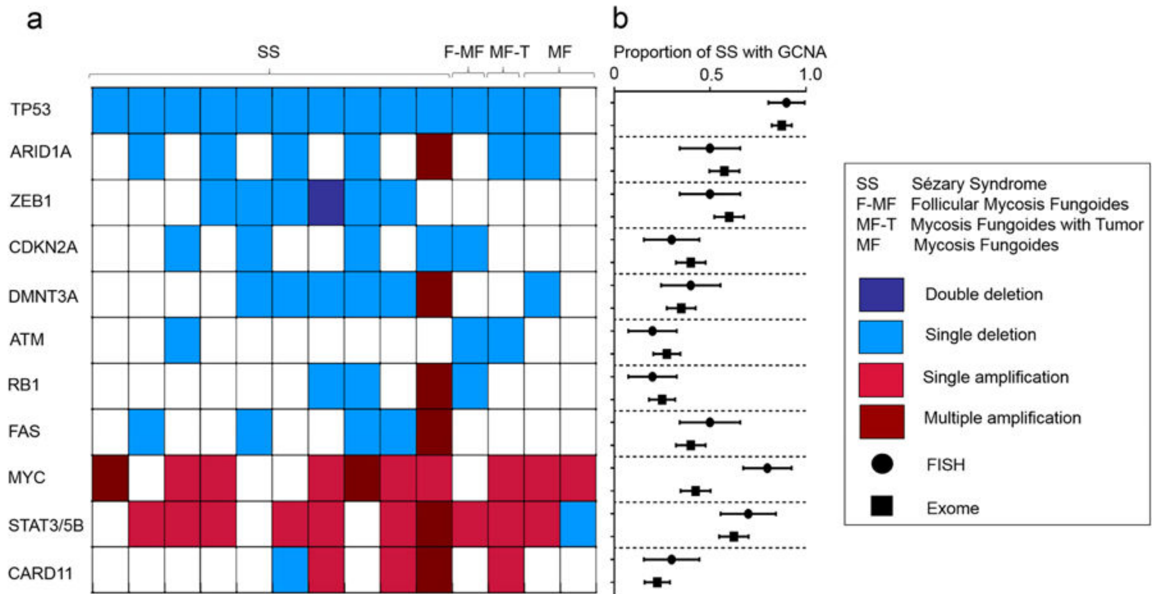

Figure 2. Distribution of deletions and amplifications detected by FISH

(**a**) Heatmap of deletions and amplification in CTCL driver genes detected by the 11-probe CTCL FISH panel among 10 patients with SS, 1 patient with F-MF, 1 patient with MF-T, and 2 patients with MF. Each column shows FISH status for one patient. (**b**) Comparison of proportion (+/− standard error) of SS patients with abnormal copy numbers in each gene by FISH (n=10) versus CTCL exome sequencing (n=40) on non-overlapping patient cohorts. No significant differences were found for any gene (Fisher's exact test, $p > 0.1$). *For STAT3/5B FISH probe, comparator gene from exome data is STAT5B.