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# Structural alterations of the FAS gene in cutaneous T-cell lymphoma (CTCL) ☆

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ABSTRACT

FAS (TNF receptor superfamily member 6, also known as CD95) plays a major role in T-cell apoptosis and is often dysregulated in CTCL. We searched for structural alterations of the FAS gene with the potential to affect its function. Although several heterozygous FAS promoter single nucleotide polymorphisms (SNPs) were detected, the only homozygous one was the −671 GG SNP present in 24/80 CTCL cases (30%). This SNP maps to an interferon response element activated by STAT-1. EMSA and supershift EMSA showed decreased CTCL nuclear protein/STAT-1 binding to oligonucleotides bearing this SNP. Luciferase reporters showed significantly less interferon-alfa responsive expression by FAS promoter constructs containing this SNP in multiple CTCL lines. Finally, FAS was upregulated by interferon-alfa in wildtype CTCL cells but not those bearing the −671 GG SNP. These findings indicate that many CTCL patients harbor the homozygous FAS promoter −671 GG SNP capable of blunting its response to interferon. This may have implications for CTCL pathogenesis, racial incidence and the response of patients to interferon-alfa therapy. In contrast, functionally significant mutations in FAS coding sequences were detected uncommonly. Among CTCL lines with the potential to serve as models of FAS regulation, FAS-high MyLa had both FAS alleles, FAS-low HH was FAS-hemizygous and FAS-negative SeAx was FAS-null.

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## Introduction

Cutaneous T-cell lymphoma (CTCL)[1] is a neoplasm of well differentiated CD4+ memory T cells belonging to the skin associated lymphoid tissue (SALT) [1]. It includes mycosis fungoides (MF) and its erythrodemic and leukemic variant, the Sézary syndrome (SS). There are multiple lines of evidence supporting the hypothesis that early CTCL is primarily a lymphoaccumulative disorder rather than a lymphoproliferative disorder, i.e., that tumor cells persist and accumulate primarily due to defective apoptosis rather than enhanced proliferation. This evidence includes the indolent clinical behavior of early CTCL, its resistance to therapy that targets rapidly proliferating cells, its relatively low proliferative rate as assessed by mitotic index or Mib-1/Ki-67 expression, and its low apoptotic rate as assessed by terminal dUTP nick-end labeling (TUNEL) assay [2,3].

One of the major systems mediating apoptotic activity in T cells is the FAS pathway [4]. FAS dysregulation by CTCL tumor cells has been reported in a variable proportion of cases using a variety of immunohistological, flow cytometric and PCR techniques [3,5–13]. In prior studies of CTCL, we determined that there is a mechanistic connection among FAS transcript level, expression of FAS protein on the cell surface, and functional sensitivity to FAS-mediated apoptosis in vitro [3]. However, structural factors affecting FAS transcript level and integrity in CTCL are largely unexplored. This set the stage for the current study in which we analyzed the primary structure of the FAS gene (see Supplementary Fig. 1) in order to search for alterations with the potential to influence FAS expression.

## Materials and methods

### *Cells and lesional tissues*

CTCL-derived (MyLa, HH, Hut-78, SeAx, SZ4, MJ) and other T-cell lines (Jurkat, JFL) were obtained from multiple sources and cultured as reported previously [3]. CTCL lesional skin and involved blood samples were obtained from our local cutaneous lymphoma tissue bank. Seven blood samples from SS were generously provided by Dr. Alain Rook (University of Pennsylvania). Specimens were collected with informed consent and Institutional Review Board approval.

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[1] *Abbreviations used*: CTCL, cutaneous T-cell lymphoma; SNP, single nucleotide polymorphisms; SS, Sézary syndrome.

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*Flow cytometry*

Surface FAS expression by T-cell lines was determined by staining with FITC or PE conjugated anti-FAS monoclonal antibody DX2 (Becton Dickinson, San Jose, CA). Isotype-matched monoclonal antibodies of irrelevant specificity were used as negative controls as described previously [3]. For interferon experiments, $2 \times 10^5$ cells were treated with 100 U/ml of interferon-α2b (Merck & Co., Inc., Whitehouse Station, NJ) for 48 h before staining for surface detection.

*Immunophenotyping*

We used a 3-stage murine monoclonal antibody/biotinylated goat anti-mouse IgG/avidin-HRP immunoperoxidase method applied to acetone-fixed frozen sections to assess CTCL skin lesions and confirm their expected CD3+4+8- helper T-cell immunophenotype. Flow cytometry was used to confirm this same immunophenotype for the leukemic blood samples.

*Cytogenetic and FISH analysis*

Cytogenetic analysis was performed as described previously [10,14]. Fluorescence in situ hybridization (FISH) analysis was performed according to the ACT Cytogenetics Laboratory Manual [14,15]. FAS probe was made using established procedures [16] by labeling the BAC Clone RP11-399O19 (chr10:90,718,801-90,775,625), which spans 56.8 kb including FAS promoter, all of the FAS exons/introns but not the nearby gene PTEN. The control probes for the 10 centromere and PTEN were purchased from Vysis (Des Plaines, IL). The 10 centromere probe hybridizes to alpha satellite sequences specific for the chromosome 10 centromere, while the PTEN probe contains sequences that span the PTEN gene at both the 5′ and 3′ ends.

*Sequencing of FAS exons and promoter*

As described previously [3], we used genomic DNA to amplify and sequence the FAS promoter and coding regions (Supplementary Fig. 1). Nucleotide numbering of the polymorphisms in the FAS promoter was based on GenBank (Accession No. X87625) and related reports [17,18]. Coding region and promoter primers are shown in Supplementary Tables 1a–1c, respectively. Some samples showing heterozygous FAS promoter polymorphisms by direct sequencing were further confirmed by DNA cloning and subsequent re-sequencing.

*Electrophoresis mobility shift assay (EMSA) and supershift EMSA*

Nuclear extracts were prepared from MyLa cells ($5 \times 10^6$/ml) using the NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce, Rockford, IL). Double-stranded oligonucleotide FAS promoter probes (−663 to −683) were chemically synthesized with wildtype A or SNP G at the −671 site. Probes were labeled using the Biotin 3′ end DNA labeling kit (Pierce, Rockford, IL). The sequence of this oligonucleotide, 5′-TGTCCATTCCAGA/GAACGTCTG-3′, contains a GAS binding site. EMSA was performed using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL). DNA-bound protein was identified by supershift EMSA with an anti-STAT-1 antibody (α-p91 and β-p84, Santa Cruz Biotechnology, Santa Cruz, CA).

*Luciferase reporter constructs*

Generation of DNA fragments with SNPs was performed using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The constructs and primers used to generate them are shown in Supplementary Table 2. Wildtype and SNP constructs were sequenced to confirm the appropriate promoter and transcription initiator sequences were present and then ligated into pGL3 luciferase basic vector. Plasmid carrying the β-galactosidase gene (2 μg/sample) was co-transfected as an internal control. Cells were stimulated by PMA (100 ng/ml) immediately after transfection. For interferon stimulation assays, $2 \times 10^6$ cells were treated with interferon-α2b (IFN) (100 U/ml) at the time of transfection.

*Statistical analysis*

Statistical analysis for the luciferase assays was performed using Student's *t*-test. A two-tailed *p*-value < 0.05 was considered statistically significant and is represented as (∗) in the figures. Analyses utilized SAS statistical software version 9.2 (SAS Institute, Inc., Cary, NC). Statistical analysis of FAS promoter genotypes among CTCL patients compared to controls was performed using the Chi-square method and two-tailed *p* values.

## Results

*Molecular analysis of the FAS promoter region in CTCL demonstrates frequent germline single nucleotide polymorphisms (SNPs)*

To search for mutations in regulatory regions of the FAS gene in CTCL, we used automated nucleotide sequencing of genomic PCR products to detect alterations of the 1781 bp FAS promoter immediately upstream of the start codon. We analyzed this region in 29 CTCL cases including 9 early MF (stages IB–IIA), 7 advanced MF and 10 SS (stages IIB–IVA), and 3 CTCL-derived cell lines: MyLa, HH and SZ4. PCR of FAS targets was negative in the SeAx CTCL line, consistent with absence of the FAS gene by FISH analysis (see below). As shown in Table 1, several single nucleotide polymorphisms (SNPs) at known germline SNP sites were detected among 24/29 CTCL cases (83%). Interestingly, each of these SNPs involved a transcription factor binding site including SP1 (−1378, RefSNP ID: rs2234767), AP1 (−1092, RefSNP ID: rs9658675), YY1 (−691, RefSNP ID: rs2234768), GAS (−671, RefSNP ID: rs1800682) and TEF (−436, RefSNP ID: rs9658676). All of these SNPs were heterozygous except for 8/29 CTCL cases (28%) that contained the homozygous −671 GG SNP. Other than these SNPs, no other mutations of the FAS promoter were identified by PCR. This indicates that somatic FAS promoter mutations are absent or rare in CTCL and do not accumulate with disease progression or in response to therapy. Consistent with our results using primarily lesional skin samples, a study of leukemic Sézary cells detected somatic mutations in the FAS promoter only very rarely [9].

In addition to searches of GenBank for FAS promoter polymorphisms, we also scanned the NCBI dsSNP database. This includes

**Table 1**
Genotypic frequencies of FAS promoter SNPs in CTCL.

| Nucleotide position (TFBS)[a] | CTCL genotypic frequencies, *N* = 29 | | |
|---|---|---|---|
| −1378 (Sp1) | GG | GA | AA |
| | 76% | 24% | 0% |
| −1092 (AP1) | GG | GA | AA |
| | 97% | 3% | 0% |
| −691 (YY1) | TT | TC | CC |
| | 90% | 10% | 0% |
| −671 (GAS) | AA | AG | GG |
| | 34% | 38% | 28% |
| −436 (TEF) | CC | CA | AA |
| | 97% | 3% | 0% |

TFBS: transcription factor binding site.
[a] TFBS were searched using search tools: TESS (Transcription Element Search System: http://www.cbil.upenn.edu/cgi-bin/tess/tess) and TRANSFAC® Public database (http://www.gene-regulation.com).

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