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Expression and Function of *FRA2*/*JUND* in Cutaneous T-Cell Lymphomas

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Abstract. *Adult T-cell leukemia/lymphoma (ATLL) and cutaneous T-cell lymphomas (CTCLs) are known to frequently express CC chemokine receptor 4 (CCR4). Previously, we investigated the transcriptional control of CCR4 expression in ATLL and have found that an activating protein 1 (AP1) family member, FBJ murine osteosarcoma viral oncogene homolog (FOS)-related antigen 2 (FRA2), is consistently expressed at high levels in ATLL and, together with v-JUN avian sarcoma virus 17 oncogene homolog D (JUND), up-regulates the expression of CCR4 as well as that of several proto-oncogenes such as v-MYB myeloblastosis viral oncogene homolog (MYB), murine double minute 2 homolog (MDM2), and B-cell lymphoma 6 (BCL6). Here, we examined the expression of these genes in clinical samples of CTCLs. We detected the transcripts of FRA2, JUND, CCR4, MYB, MDM2, and BCL6 at high levels in CTCL skin lesions. Except for BCL6, we confirmed protein expression of FRA2, JUND, CCR4, MYB, and MDM2 in CTCL skin lesions. Furthermore, siRNA-mediated knockdown of FRA2 or JUND suppressed cell growth and the expression of CCR4, MYB, MDM2, and BCL6 in CTCL cell lines. Our results, thus, demonstrate the presence of a common oncogenic cascade initiated by FRA2/JUND in CCR4-expressing mature T-cell malignancies such as ATLL and CTCLs.*

Adult T-cell leukemia/lymphoma (ATLL) is an aggressive mature cluster of differentiation 4 $(CD4)^+$ T-cell malignancy etiologically associated with human T-cell leukemia virus type 1 (HTLV-1) (1). Previously, we and others have shown that primary leukemia cells from most cases of ATLL strongly express CC chemokine receptor 4 (CCR4) (2-4). CCR4 is a chemokine receptor known to be selectively expressed by helper T2 (Th2) cells, forkhead box P3 $(FOXP3)^+$ regulatory T-cells, and cutaneous lymphocyte-associated antigen $(CLA)^+$ skin-homing memory T-cells (5). Therefore, CCR4 expression may be related to the origin of ATLL and may also account for the frequent skin involvement of ATLL (2, 3). We have further analyzed transcriptional control of CCR4 expression in ATLL and found that *FBJ murine osteosarcoma viral oncogene homolog (FOS)-related antigen 2* (*FRA2*), a member of the FOS family activator protein-1 (AP1) transcription factors, which is hardly expressed at all in normal mature $CD4^+$ T-cells, is constitutively expressed in primary leukemia cells of ATLL, and together with *v-JUN avian sarcoma virus 17 oncogene homolog D* (*JUND*), a member of the JUN family AP1 transcription factors, which is constitutively expressed even in normal mature $CD4^+$ T-cells, is responsible for high-level expression of *CCR4* (6). The AP1 family transcription factors are involved in various important cellular processes including oncogenesis and function as dimers: either heterodimers between the FOS (c-FOS, FOSB, FRA1, and FRA2) and JUN family members (c-JUN, JUNB, and JUND) or homodimers of the JUN family members (7, 8). Indeed, we have further shown that *FRA2* and *JUND* cooperatively promote cell proliferation and up-regulate the expression of several proto-oncogenes such as *v-MYB myeloblastosis viral oncogene homolog* (*MYB*), *murine double minute 2 homolog* (*MDM2*), and *B-cell lymphoma 6* (*BCL6*) in ATLL (6).

Cutaneous T-cell lymphomas (CTCLs) are a heterogeneous group of mature T-cell lymphomas including mycosis fungoides (MF), Sézary syndrome (SS), and anaplastic large cell lymphoma (ALCL) (9). CTCLs are not etiologically associated with HTLV-1. However, CTCLs are also known to frequently express CCR4, albeit less strongly than ATLL (9-12). CTCLs have also been reported to overexpress *MYB*, which is detected early in the disease development (13). Thus, *FRA2*/*JUND* may also play an oncogenic role in CTCLs. We indeed detected strong expression of *FRA2* and *JUND* in CTCL cell lines (6).

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Table I. *Summary of skin tissues used in this study.*

Case		Gender	Years	Diagnosis
Normal	1	Male	78	Extramammary Paget's disease (abdomen)
	2	Male	83	Extramammary Paget's disease (abdomen)
	3	Male	66	Extramammary Paget's disease (abdomen)
	4	Female	51	Extramammary Paget's disease (abdomen)
	5	Female	78	Extramammary Paget's disease (abdomen)
Atopic dermatitis	1	Female	31	Acute eczematous lesion
	2	Male	35	Acute eczematous lesion
	3	Male	55	Acute eczematous lesion
Mycosis fungoides	1	Male	75	Tumor stage
	2	Female	72	Tumor stage
	3	Male	82	Tumor stage
	4	Female	23	Tumor stage
	5	Female	74	Tumor stage
	6	Male	68	Tumor stage
	7	Female	65	Tumor stage
Anaplastic large cell lymphoma	1	Male	62	$CD30^+$
	2	Male	78	$CD30^+$
	3	Female	58	$CD30^+$
	4	Male	80	$CD30^+$
	5	Male	61	$CD30^+$

However, the expression of *FRA2* and *JUND* and their presumed downstream target genes has not been studied in clinical samples of CTCLs.

In this study, therefore, we examined expression of *FRA2* and *JUND* and their potential target genes in clinical samples of CTCLs. We also examined the role of FRA2 and *JUND* in CTCL cell lines by siRNA knockdown experiments. Our results demonstrate a highly similar oncogenic role of *FRA2*/*JUND* in ATLL and CTCLs.

Materials and Methods

Cell lines. HUT78 (a $CD4^+$ T-cell line derived from SS), HH (a $CD4^+$ T-cell line derived from non-MF/SS aggressive CTCL) and MyLA (a $CD8^+$ T-cell line derived from MF) were obtained from the Health Science Research Resources Bank (Osaka, Japan). HSB-2 (a T-ALL cell line) was routinely maintained in our laboratory (2). All these cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum.

Polymerase chain reaction. Formalin-fixed, paraffin-embedded tissue sections of normal skin samples (n=5), skin lesions of atopic dermatitis (AD) (n=3), skin lesions of mycosis fungoides (MF) (n=7), and skin lesions of anaplastic large cell lymphoma (ALCL) (n=5) were obtained from the Department of Pathology, Kinki University Faculty of Medicine (Table I). This study was approved by the Ethical Committee of Kinki University Faculty of Medicine. Total RNAs were extracted from cell lines and tissue sections as described previously (14). Semi-quantitative Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR were performed as described previously (6). The primers and fluorogenic probes for quantitative real-time PCR were obtained from TaqMan kit (Applied Biosystems, Foster City, CA, USA).

Immunohistochemistry. Tissue sections from MF patients (n=4) and ALCL patients (n=3) were treated in a target retrieval solution (DAKO, Kyoto, Japan), incubated with anti-CCR4 (KM-2160; Kyowa Hakko, Tokyo, Japan), anti-MYB (4E3; Calbiochem, La Jolla, CA, USA), anti-MDM2 (1B10; Novocastra Laboratories, Newcastle upon Tyne, UK), anti-BCL6 (PG-B6p; DAKO), rabbit polyclonal anti-FRA2 (sc-604; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal anti-JUND (sc-74; Santa Cruz Biotechnology). Isotype-matched mouse IgG (DAKO) and normal rabbit IgG (DAKO) were used as negative controls. Then, the tissue sections were washed, sequentially incubated with biotin-labeled secondary antibodies and avidin-biotin complex/horseradish peroxidase (Vectastain ABC/HRP kits; Vector Laboratories, Burlingame, CA, USA) and counterstained with Gill's hematoxylin.

Transfection. Small-interfering RNAs (siRNAs) for FRA2 (SI00420455), JUNB (SI03077445), JUND (SI00075985) and negative control siRNA (1022064) were obtained from Qiagen (Hilden, Germany). Transfection was performed on Amaxa Nucleofector (Amaxa, Cologne, Germany) using T solution and O-17 program. The transfection efficiency was close to 95%, as determined by fluorescent siRNA (Qiagen). The transfected cells were seeded in a 96-well plate (0.5×10^4 cells/well), and viable cell count was performed every 24 h on a FACSCalibur instrument (Becton Dickinson, Mountain View, CA, USA) by gating out dead cells stained with propidium iodide.

Statistical analysis. Student's *t*-test was used for statistical analysis, considering $p<0.05$ as being statistically significant.

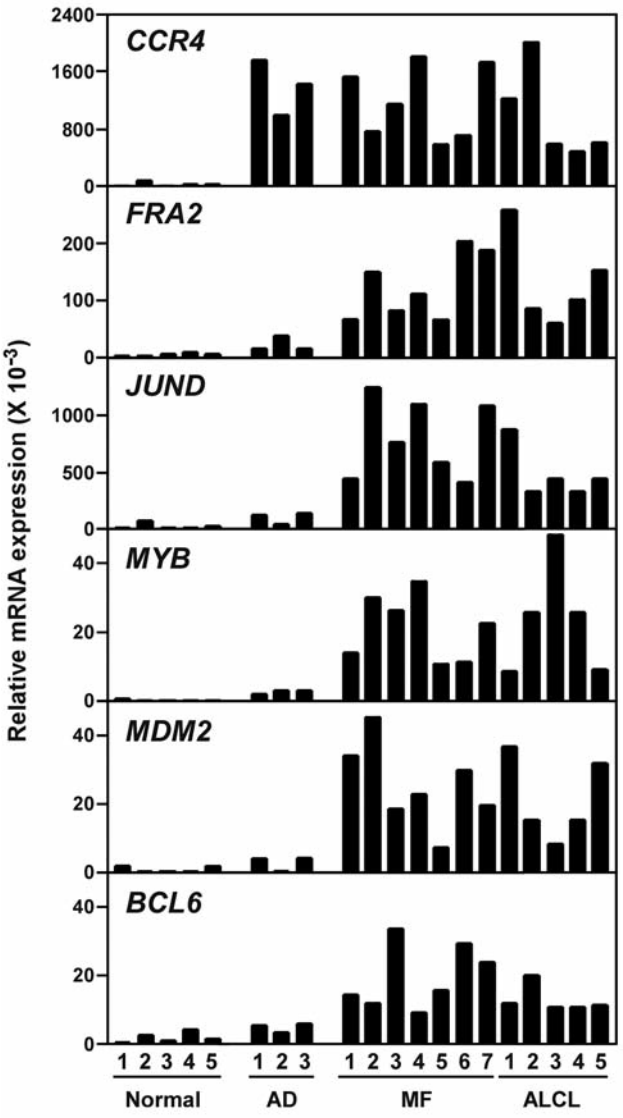

Figure 1. *mRNA expression in skin tissues. Total RNAs were extracted from formalin-fixed, paraffin-embedded sections of normal skin samples (n=5), skin lesions of atopic dermatitis (AD) (n=3), skin lesions of mycosis fungoides (MF) (n=7), and skin lesions of anaplastic large cell lymphoma (ALCL) (n=5). Quantitative real-time polymerase chain reaction (PCR) was performed for CC chemokine receptor 4 (CCR4), FBJ murine osteosarcoma viral oncogene homolog (FOS)-related antigen 2 (FRA2), v-JUN avian sarcoma virus 17 oncogene homolog D (JUND), v-MYB myeloblastosis viral oncogene homolog (MYB), murine double minute 2 homolog (MDM2), and B-cell lymphoma 6 (BCL6). Levels of expression are shown relative to that of β2-microglobulin used as the internal control. Representative results from two separate experiments are shown.*

Results

Our previous study has shown that an AP1 member *FRA2* is highly expressed in primary leukemia cells of ATLL (6). Furthermore, we have shown that *FRA2* in conjunction with *JUND* is involved in cell proliferation and is responsible for the up-regulation of genes such as *CCR4, MYB, MDM2*, and *BCL6* (6). We also detected high-level expression of *FRA2, JUND, MYB, MDM2,* and *BCL6* in CTCL cell lines (data not shown). Therefore, we first examined the expression of these six genes in skin lesions of CTCLs. We first performed quantitative real-time PCR using total RNA samples extracted from paraffin-embedded skin sections (Table I). As shown in Figure 1, the transcripts of *FRA2* and *JUND*, together with those of *CCR4*, were indeed detected in skin lesions of MF (n=7) and ALCL (n=5) at much higher levels than those of normal skin samples (n=5) or skin lesions of atopic dermatitis (AD) (n=3). Although AD skin lesions contained *CCR4* transcripts at high levels, consistent with the abundant infiltration of Th2 cells, the transcripts of *FRA2* and *JUND* were not elevated. Furthermore, the transcripts of *MYB, MDM2*, and *BCL6*, the presumed downstream target genes of *FRA2*/*JUND* (6), were also detected at high levels in CTCL skin lesions.

We next performed immunohistochemical staining of FRA2, JUND, CCR4, MYB, MDM2, and BCL6 in skin lesions from patients with MF (n=4) and ALCL (n=3). Representative results are shown in Figure 2. FRA2, JUND, CCR4, MYB, and MDM2 were indeed consistently stained in tumor cells of all cases of MF and ALCL examined. However, BCL6 was hardly stained in tumor cells of any CTCL skin lesions. We also detected MYB and MDM2 but not BCL6 in leukemia cells of ATLL skin lesions (data not shown). Therefore, *BCL6* mRNA may not be efficiently translated to the protein in ATLL and CTCLs.

To examine the possible oncogenic role of *FRA2* and *JUND* in CTCLs, we next performed siRNA knockdown experiments using two CTCL cell lines HUT78 and MyLa. As shown in Figure 3A, *FRA2* siRNA, *JUNB* siRNA, and *JUND* siRNA specifically reduced the levels of respective transcripts in these two CTCL cell lines. On the other hand, control siRNA had no such effect. Under these conditions, we first examined cell growth. As shown in Figure 3B, *FRA2* siRNA and *JUND* siRNA, but not *JUNB* siRNA or control siRNA, significantly ($p<0.05$) reduced cell growth in both CTCL cell lines. On the other hand, *FRA2* siRNA or *JUND* siRNA hardly affected at all the growth of control HSB-2 cells, a T-ALL cell line. We also compared the effect of single and double knockdown of *FRA2* and *JUND* on cell growth (Figure 3C). Compared to the significant ($p<0.05$) growth suppression by single knockdown of *FRA2* and *JUND*, no further inhibition was observed by knockdown of both *FRA2* and *JUND* in either cell line. These results were consistent with the notion that *FRA2* and *JUND* promote the growth of CTCL cell lines by working together, *i.e.* by forming the heterodimer.

We next examined the expression of *CCR4, MYB, MDM2* and *BCL6* by quantitative RT-PCR. As shown in Figure 3D, *FRA2* siRNA and *JUND* siRNA, but not *JUNB* siRNA or

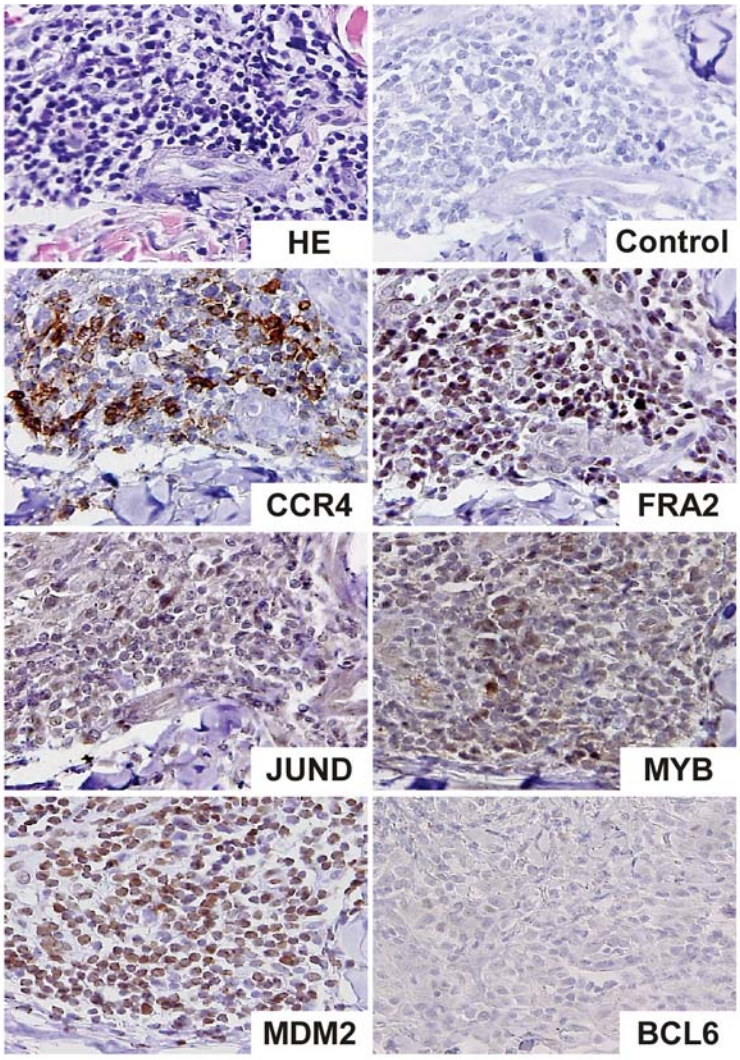

Figure 2. *Protein expression in skin tissues. Tissue sections from patients with mycosis fungoides (MF) (n=4) and anaplastic large cell lymphoma (ALCL) (n=3) were stained with anti-CCR4, anti-FRA2, anti-JUND, anti-MYB, anti-MDM2, and anti-BCL6. Mouse IgG1 and normal rabbit IgG were used as negative controls (control). The tissue sections were counterstained with Gill's hematoxylin. The representative results from one patient with MF are shown. Original magnification, ×400.*

control siRNA, significantly ($p<0.05$) reduced the expression of *CCR4, MYB, MDM2*, and *BCL6* by approximately 50% in both cell lines. These results indicate that *CCR4, MYB, MDM2, BCL6* are downstream target genes of *FRA2* and *JUND* in CTCLs as in ATLL (6).

Disucussion

FRA2 was originally identified as a gene encoding FOS-related antigen gene-2 (15, 16) and shown to be oncogenic for chicken embryonic fibroblasts (CEFs) (16). *FRA2* was also found to be an essential partner for the oncogenic activity of spontaneously isolated *JUND* mutants in CEFs (17). In human breast cancer, *FRA2* expression has been reported to enhance invasive properties (18, 19), to induce bone sialoprotein expression (20), and to correlate with nodal involvement and reduced disease-free survival (19). Previously, we have shown that *FRA2* is consistently expressed at high levels in primary leukemia cells of ATLL, while normal resting $CD4^+$ T-cells, mitogen-activated $CD4^+$ T-cells or $CD4^+$ T-cells polarized to Th1 or Th2 hardly expressed *FRA2* at all (6). Furthermore, we have shown that *FRA2*, together with *JUND*, which is constitutively expressed in normal $CD4^+$ T-cells, is involved in cell growth of ATLL cell lines and responsible for the high-level expression of *CCR4* and also for the up-regulation of several proto-oncogenes such as *MYB, MDM2*, and *BCL6* (6). In this study, we have further shown that *FRA2* and *JUND* are

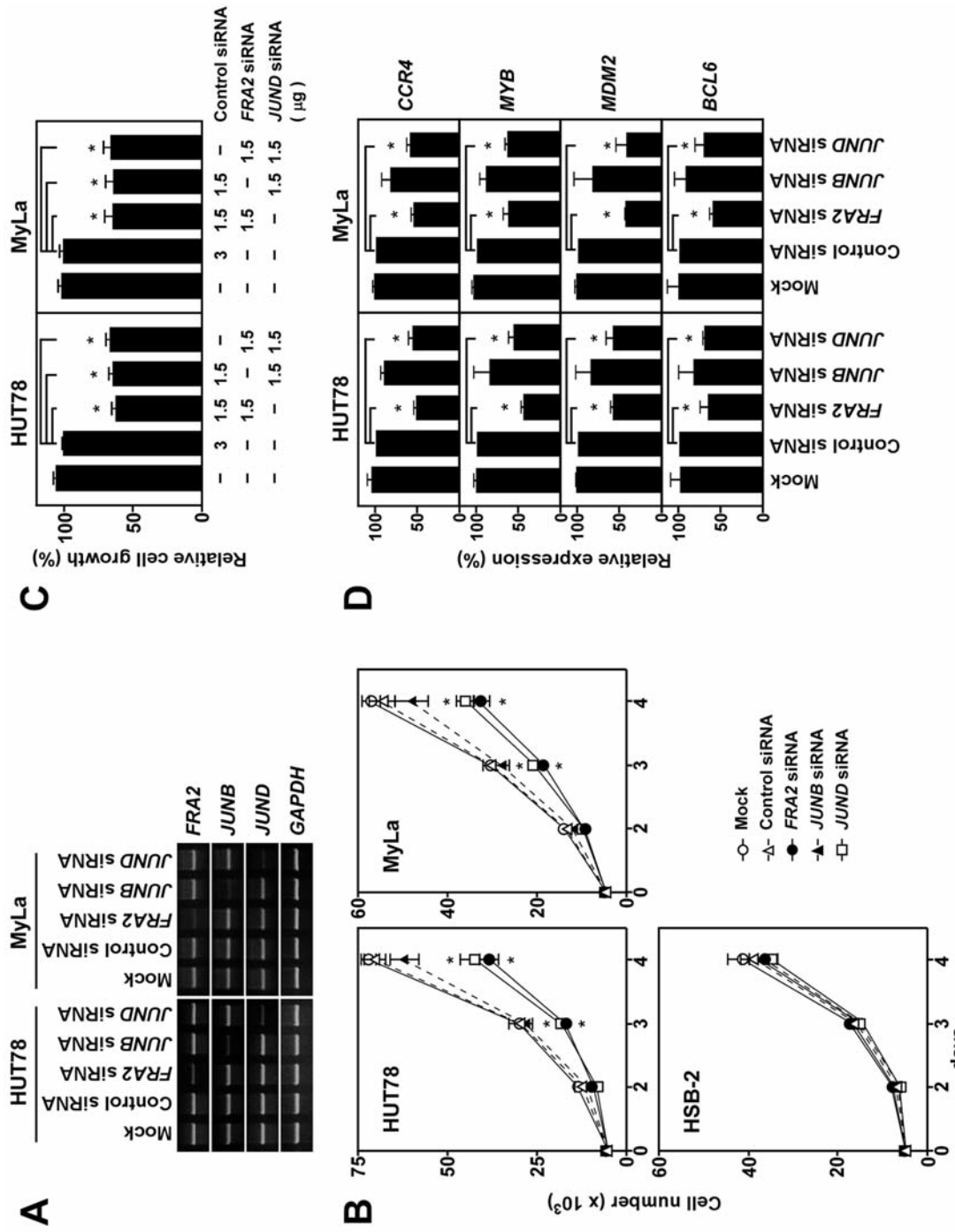

Figure 3. *siRNA knockdown experiments. A: Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Two CTCL cell lines HUT78 and MyLa were transfected with 2.5 μg of control, FRA2, JUNB, or JUND siRNA. After 48 h, total RNAs were extracted. RT-PCR was performed for the transcripts of FRA2, JUNB, JUND, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The representative results from three separate experiments are shown. B: Cell growth analysis. HUT78, MyLa, and HSB-2 (a control T-ALL cell line) were transfected with 2.5 μg of control, FRA2, JUNB, or JUND siRNA, and cultured in a 96-well plate at 0.5×10⁴ cells/well. At the indicated time points, viable cell numbers were determined on a FACSCalibur instrument by gating out cells stained with propidium iodide. Data are shown as the mean±SEM from three separate experiments. *p<0.05. C: Double knockdown experiments. HUT78 and MyLa were transfected with siRNAs for the control, FRA2, or JUND as indicated, and cultured in a 96-well plate at 0.5×104 cells/well. After 4 days, viable cell numbers were determined on a FACSCalibur instrument by gating out cells stained with propidium iodide. Data are shown as the mean±SEM from three separate experiments. *p<0.05. D: Quantitation of transcripts. Real-time quantitative PCR was performed to quantitate the transcripts of CCR4, MYB, MDM2, and BCL6 relative to β2-microglobulin used as an internal control. Data are shown as the mean±SEM from three separate experiments. *p<0.05.*

consistently expressed at elevated levels in clinical samples of CTCLs (Figures 1 and 2) and also involved in cell growth and up-regulation of *CCR4, MYB, MDM2*, and *BCL6* in two CTCL cell lines (Figure 3). The present results obtained from CTCLs are thus highly similar to those obtained from ATLL (6), supporting the presence of a common oncogenic cascade initiated by *FRA2*/*JUND* in CCR4-expressing mature T-cell malignancies such as ATLL and CTCLs.

MYB is a proto-oncogene implicated in human malignancies, such as acute myeloid leukemia, chronic myeloid leukemia, a subset of T-ALL, colon cancer, and breast cancer (21). *MYB* is also known to be highly expressed in CTCLs (13, 22). Previously, interleukin (IL) -7 and IL-15 were shown to induce the expression of *MYB* in an IL-7- and IL-15-dependent CTCL cell line (22). Our present results have further demonstrated that MYB is a downstream target gene of *FRA2* and *JUND* in CTCLs.

MDM2 encodes an E3 ubiquitin ligase, which is a key negative regulator of p53 (23). In CTCLs, mutations affecting *p53* are rare and only seen in advanced stages (9, 24), when CCR4 expression by tumor cells also tend to be decreased (25). Thus, the elevated expression of *MDM2* by *FRA2* and *JUND* may be partially responsible for functional down-regulation of p53 in the early stages of CTCLs.

BCL6 is an anti-apoptotic protein frequently up-regulated in diffuse large B-cell lymphoma and follicular B-cell lymphoma (26). In T-cells, follicular helper T-cells and angioimmunoblastic T-cell lymphoma are known to express *BCL6* (27). Our immunohistochemical studies, however, failed to prove the protein expression of *BCL6* in CTCLs (Figure 2) and ATLL (data not shown). Thus, the up-regulated expression of *BCL6* mRNA may not be efficiently translated to BCL6 protein in ATLL and CTCLs.

We still do not know how the expression of *FRA2* is up-regulated in these mature T-cell malignancies. In this context, although the FOS family genes are categorized as immediate early genes that respond quickly and transiently upon various stimuli, *FRA2* is known to be unique for its delayed and prolonged expression upon induction (15, 28, 29). Indeed, *FRA2* expression was shown to be indirectly induced by *c-FOS* in chicken embryo fibroblasts upon serum stimulation and then maintained by FRA2 itself through activation by phosphorylation (30-32). Therefore, such a positive feedback mechanism may be partly responsible for the constitutive expression of *FRA2* in ATLL and CTCLs.

Clinical trials employing a humanized antibody to CCR4 are ongoing for ATLL in Japan and CTCLs in U.S.A. with promising results (33, 34). In this context, our findings that *CCR4* is one of the downstream target genes of *FRA2* and *JUND* and thus could be regarded as an authentic tumor-associated antigen in ATLL and CTCLs, provide a strong rational basis for anti-CCR4 therapy. *FRA2* and *JUND* themselves may also provide novel diagnostic and therapeutic targets for these T-cell malignancies. The roles of various proto-oncogenes up-regulated by *FRA2*/*JUND* in these T-cell malignancies remain to be elucidated.

Conflict of Interest Statement

We have no conflicts of interest to declare.

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