別紙１

厚生労働科学研究費補助金

がん臨床研究事業

原発不明がんの診断・効果的治療の確立に関する研究

平成１９年度　総括研究報告書

主任研究者　中川　和彦

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主任研究者　中川　和彦
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研究要旨　　臓器別体系を機軸とした我国のがん診療体制における原発不明がんの治療は不適切、かつ遅延することが多い。原発不明がんを対象とした臨床試験の実施により、原発不明がんの診断と治療に関する基本的方針の啓蒙を図る。またDNA発現解析により原発巣の推定を行う新しい治療戦略の画一的な従来の原発不明がん治療戦略に対する臨床的有用性を問う第III相比較試験の実施妥当性を無作為化臨床第II相試験にて評価する。

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Ａ．研究目的

原発不明がんを対象とした臨床試験の実施により、原発不明がんの診断と治療に関する基本的方針の啓蒙を図る。またDNA発現解析により原発巣の推定を行う新しい治療戦略の画一的な従来の原発不明がん治療戦略に対する臨床的有用性を問う第III相比較試験の実施妥当性を無作為化臨床第II相試験にて評価する。

Ｂ．研究方法

①第一段階：研究組織と運営組織の確立

1）研究組織：現状では腫瘍内科を有する医療施設は少ない。当初、申請書に示した参加施設で共同研究組織を設立、プロトコール作成を開始する。その後、日本全国のがん薬物専門医に研究協力者を募る。遺伝子発現解析による原発巣の推定には、既存の遺伝子発現解析結果を有する基礎研究者、解析結果から原発巣を推定するアルゴリズムを構築する生物統計家の協力体制を確立する。

2）運営組織：本研究の運営組織として、委託契約を締結して非営利活動法人西日本胸部腫瘍臨床研究機構（NPO-WJOG）のデータセンター機能と効果安全性評価委員会による外部評価機能を使用する。また、国立がんセンター中央病院の病理研究者の本研究への協力を求め、病理診断の中央判定の実施を可能とする。このことにより参加施設の病理診断技術レベルの改善を図る。

3）遺伝子発現解析データベースに基づく原発巣推定のアルゴリズム：近畿大学ゲノム生物学教室西尾教授の保持する過去の遺伝子発現解析データを用いて、アルゴリズム作成とその検証を東京大学伊藤先生らにより作成、検証される。

②第二段階：臨床試験実施計画書の作成と対象患者選択方法の確立

1）臨床試験プロトコールの作成：臨床試験デザインに関しては参加施設の合意形成が重要である。これまでの原発不明癌を対象にした臨床試験（phase II studyばかりであるが）において、プラチナ製剤を含む化学療法での生存期間中央値は6-10か月と報告されている。したがって1年生存率は35%と仮定する。それに対して今回、DNAチップを用いて原発巣を推定することでより個々の症例において標準的治療法を受ける可能性が高いものと推定し、1年生存率を50%と仮定した。βエラーを0.02、αエラーを0.05とすると登録期間3年、追跡期間2年とした場合、各群77例必要となる。逸脱例も考慮してtotal 160例必要となる。現在の参加施設（12施設）の患者集積力は年間80症例であることから本試験は実施可能である。原発不明がんの診療指針の啓蒙のために、今後、更に参加施設を追加する。

2) 対象患者選択方法の確立：本研究参加施設の中でも考え方の相違が存在する。病理診断を含めた医学情報に基づいて「予後良好な原発不明がん」を除外する統一基準を作成する。

3) 試験開始に当たっては、参加施設、班長協力者に集まって頂き、キックオフ・ミーティングを開催する。

③第三段階：臨床試験の実施

1) 症例登録とランダム割付：WJOGデータセンターでの中央登録方式とする。登録票記入後、データセンターへFAXにて登録、データセンターより配布された患者識別番号を用いて臨床検体を三菱安全科学研究所へ送付する。遺伝子発現結果は近畿大学医学部ゲノム生物学教室に送られ、完成されたアルゴリズムを用いて原発巣を推定する。推定結果はWJOGデータセンターに送られ、データセンターは割付結果を実施施設に通知する。

2) 治療方法：
対照治療群：カルボプラチンとパクリタキセルの2剤併用療法
試験治療群：遺伝子発現解析にて推定された原発巣のあらかじめ定められた標準的治療を実施する。

3) 予定症例数：160症例（各群80症例）

④実施期間と年次計画

1) 一年次：第一、第二段階で示す臨床試験実施の準備を行う。
2) 二年・三年次：第三段階であるランダム化臨床第III相比較試験を開始する。中間
解析、定期モニタリングの実施。
3) 三年次：最終解析

（倫理面への配慮）
本研究では、抗癌剤感受性の高い予後良好な原発不明がん患者が本研究から最大限除外されるよう配慮する。さらに、ヘルシンキ宣言およびわが国の「臨床研究に関する倫理指針」に従い、以下の事項を厳守する。
①研究実施計画書をWJOGプロトコール審査委員会で審査し、各施設のIRB承認の得られた施設のみ症例登録を可能とする。
②全ての患者に説明文書を用いて十分な説明を行い、考慮の時間を設けた後に患者自身の自由意志による同意を文書で取得する。
③データの取り扱いに関して、直接個人を識別できる情報を用いず、データベースのセキュリティを確保し、個人情報の保護を厳守する。
④プロトコール審査委員会、効果・安全性評価委員会を組織し、研究の第三者的監視を行う。
⑤本解析でおこなうマイクロアレイによる遺伝子発現解析はヒトゲノム・遺伝子解析研究に関する倫理指針の対象ではないが、指針の趣旨を尊重し、準じた管理を行うことにより個人情報等倫理的に十分に配慮する。

C．研究結果
＜国内・国外における研究状況＞
①予後不良な「狭義の原発不明がん」に対して海外で実施された臨床第II相試験の多くはプラチナ製剤と新規抗がん剤を併用した化学療法であり、それらの奏効率は22%から55%、MSTは6ヶ月から13ヶ月と報告されている（Hainsworth JD, et al: J Clin Oncol 15: 2385-2393, 1997, Greco FA, et al: J Clin Oncol 20: 1651-1656, 2002, Culine S, et al: J Clin Oncol 21: 3479-3482, 2003）。これらの中で、比較的良好な成績を示したものはプラチナ製剤とタキサン系薬剤の2剤併用療法であった。現在、カルボプラチンとパクリタキセルの2剤併用療法が原発不明がんに対して最も汎用されている治療法である。
②国内での臨床試験は、シスプラチン＋ドセタキセル併用療法の臨床第II相試験のみである。奏効率57%、生存期間中央値12か月と良好な成績を示した（松本光史、他：第4回日本臨床腫瘍学会総会学会誌 p176, 2006）。
③フランスではシスプラチン＋ゲムシタビンとシスプラチン＋CPT-11の比較第II相試験結果に基づき、シスプラチン単剤に対するシスプラチン＋ゲムシタビン併用療法の優位性を検証する臨床第III相試験が実施されている。
④遺伝子発現解析による癌種、組織型の診断技術は近年顕著な発展を示している。原発不明がんの遺伝子発現解析も実施され臨床応用が期待されている。
＜この研究の特色・独創的＞
本研究は、現行の画一的な治療戦略（本研究では、カルボプラチンとパクリタキセルの2剤併用療法）と比較して、遺伝子発現解析により推定された癌種として個別に治療方針を決定する新しい治療戦略の臨床的有用性を評価する先進的な研究であり、世界的にも極めて価値が高い。

D．考察

＜臨床試験を企画・実施すること自体の必要性と期待される成果＞

「原発不明がん」は臓器横断的診療体制を採る診療科（腫瘍内科）でなければ適切な診断・治療ができない象徴的な疾患である。我国の中核病院に臓器横断的診療体制を推進し、それを担う腫瘍内科医を育成するためには、がん臨床医が興味を示す優れた臨床研究を実施すること必要である。臨床試験の実施により、「広義の原発不明癌」の中から予後良好な患者群を適切・迅速に選別し、最も効果的な標準治療を実施することにより原発不明がん治療の成績向上が期待できる。

＜臨床試験結果の必要性と結果から期待される成果＞

原発不明がんに対する現行の画一的な治療戦略から、遺伝子発現解析による原発巣の推定を通して、原発不明癌患者に対する個別化治療という新しい治療戦略への転換を促すことが期待される。

E．結論

本年度はプロトコールデザインの修正とそれに伴う必要事項（遺伝子発現解析アルゴリズム）の確定に時間と労力を費やした。その結果、班員の了解を得られるプロトコールを完成することができた。次年度は早急にプロトコールを実施に移し、臨床試験の完遂を目指したい。

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G．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

別紙４

研究成果の刊行に関する一覧表レイアウト（参考）

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CASE REPORT

A case of respiratory akathisia in a cancer patient: A case report

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ABSTRACT

Objective: It has been reported that akathisia is a neurological side effect induced by antiemetic drugs and/or antipsychotics. Akathisia can occur in any area of the body, but respiratory akathisia is an unusual type of akathisia. Cases of respiratory akathisia in cancer patients taking antiemetic drugs have not previously been reported.

Methods: We report on a case of a cancer patient taking prochlorperazine as an antiemetic drug who experienced dyspnea accompanied by severe restlessness associated with respiration. By administration of biperiden, his restlessness in respiration and dyspnea promptly disappeared.

Results: This finding led us to conclude that this cancer patient was experiencing respiratory akathisia.

Significance of results: Respiratory akathisia is uncommon. It is important for cancer patients that dyspnea induced by disease progression be ruled out as a cause of the respiratory restlessness. It is necessary to consider the possibility of akathisia in patients that complain of vague anxiety, chest discomfort, or dyspnea following antipsychotic medication.

KEYWORDS: Respiratory akathisia, Cancer, Antiemetic drug

INTRODUCTION

Akathisia is a neurological side effect produced by antipsychotic or antiemetic drug therapy (Blaisdell, 1994). The clinical picture of akathisia is a feeling of inner restlessness in the limbs, especially in the legs (Gibb & Lee, 1986). However, reports have indicated that akathisia can occur in any area of the body, such as the arms or abdomen (Raskin, 1972; Ratey & Salzman, 1984; Walters et al., 1989). A rare manifestation of akathisia reported by patients receiving antipsychotic treatment is an inner restlessness in respiration as dyspnea.

Prochlorperazine is an antiemetic agent frequently used by cancer patients taking opioids (e.g., morphine, oxycodone) for cancer pain. In oncological settings, prochlorperazine is used as an antiemetic drug for nausea, a side effect of opioid. It is a phenothiazine antiemetic that has central dopamine antagonist properties and that has been reported to cause acute extrapyramidal side effects,

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parkinsonism, dystonia, and akathisia (Bateman et al., 1989). It is well known that neuroleptic-induced akathisia may be difficult to recognize and can occur in the absence of other extrapyramidal signs. Furthermore, cases of akathisia due to antiemetic drugs used by cancer patients have been little reported.

CASE REPORT

The patient was an 66-year-old man with squamous cell carcinoma of the esophagus, stage II(T2N0M0). Due to his renal impairment and the presence of emphysema, surgical resection was not performed; furthermore, chemotherapy was not indicated. Therefore, he attempted radiation therapy and received a total dose of 70.2 Gy. He used opioid, 20 mg/day of morphine hydrochloride, for pain of esophagitis by irradiation, with taking prochlorperazine as an antiemetic drug. He complained of chest discomfort after receiving 5 mg/day of prochlorperazine p.o. for 3 weeks and was admitted to the hospital. When he arrived in the hospital, he acknowledged dyspnea with vague anxiety and a subjective restlessness in respiration, with a temperature of 36.8°C, blood pressure of 118/72 mm Hg, pulse 79 beats/min, respiratory rate 18 breaths/min. Resting room-air oxygen saturation was 98%. First, radiation pneumonitis was suspected, but chest X-ray was normal. He felt that he could not respire leisurely nor stop breathing at any time because of this restlessness in respiration. He denied restlessness in the limbs or other body areas except for the chest. He showed no signs or symptoms of parkinsonism. He was administered 5 mg of biperiden d.i.v.; his restlessness in respiration and dyspnea simultaneously disappeared approximately 1 h later (Hirose & Ashby, 2000). Subsequently, 6 mg of oral biperiden was added to the treatment regimen. The next day, the dyspnea with vague anxiety and other restless movements completely ceased. No signs or symptoms of akathisia have appeared in this patient since that time.

DISCUSSION

We reported respiratory akathisia in cancer patients taking prochlorperazine as antiemetics. This is the first report of respiratory akathisia recognized in cancer patients.

It was necessary that other medical problems known to produce dyspnea, such as panic attacks and dyskinesia and dystonia or pulmonary diseases, could be ruled out as a cause of the respiratory restlessness (Hirose, 2000). In this case, the patient did not have anxiety about dying or a history of panic disorder before. Respiratory dyskinesia presents as involuntary movements of respiratory muscles, but not as a restless feeling in respiration, and is not improved on treatment with biperiden (Kruk et al., 1995; Esmail et al, 1999; Heard et al., 1999). Furthermore, in this case, dystonia was ruled out by the absence of tonic contractions of respiratory muscles (Dressler & Benecke, 2005).

Respiratory akathisia is uncommon, so one needs to ask specific questions about restlessness in breathing to recognize this type of akathisia. Therefore, if physicians is not aware of inner restlessness in respiration, it is possible that dyspnea in akathisia may be overlooked or misdiagnosed as a symptom of anxiety disorders, agitation, or respiratory symptoms of cancer itself (Hirose, 2000).

Antiemetics possessing a central antidopaminergic effect are suspected to have caused the akathisia (Seeman, 2002; Matsui-Sakata et al., 2005). Antiemetic-induced akathisia has been reported in cancer patients receiving metoclopramide or prochlorperazine to help control chemotherapy-related nausea and vomiting (Fleishman et al., 1994; Tsuji et al., 2006). In this case, prochlorperazine was used as an antiemetic drug for nausea and vomiting, a side effect of opioid.

Prochlorperazine is a phenothiazine antiemetic that has central dopamine antagonistic properties. It has been reported that the presumed community standard of prescribing prochlorperazine, dexamethasone, or a 5HT3 receptor antagonist after moderately high to highly emetogenic chemotherapy results in equivalent outcomes in terms of control of vomiting and measures of satisfaction and quality of life (Burris et al., 1996; Crucitt et al., 1996).

In Japan, many cancer patients taking opioids for cancer pain clinically use prochlorperazine as an antiemetic drug. Therefore, it should be noted that akathisia is considered a possible side effect during the management of cancer pain.

The clinicians' attitude toward akathisia is important to recognize. It is also important to consider the possibility of akathisia in patients that complain of vague anxiety, chest discomfort, or dyspnea following antipsychotic medication.

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ORIGINAL ARTICLE

Aberrant expression of Fra-2 promotes CCR4 expression and cell proliferation in adult T-cell leukemia

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Adult T-cell leukemia (ATL) is a mature CD4+ T-cell malignancy etiologically associated with human T-cell leukemia virus type 1 (HTLV-1). Primary ATL cells frequently express CCR4 at high levels. Since HTLV-1 Tax does not induce CCR4 expression, transcription factor(s) constitutively active in ATL may be responsible for its strong expression. We identified an activator protein-1 (AP-1) site in the CCR4 promoter as the major positive regulatory element in ATL cells. Among the AP-1 family members, Fra-2, JunB and JunD are highly expressed in fresh primary ATL cells. Consistently, the Fra-2/JunB and Fra-2/JunD heterodimers strongly activated the CCR4 promoter in Jurkat cells. Furthermore, Fra-2 small interfering RNA (siRNA) or JunD siRNA, but not JunB siRNA, effectively reduced CCR4 expression and cell growth in ATL cells. Conversely, Fra-2 or JunD overexpression promoted cell growth in Jurkat cells. We identified 49 genes, including c-Myb, BCL-6 and MDM2, which were downregulated by Fra-2 siRNA in ATL cells. c-Myb, BCL-6 and MDM2 were also downregulated by JunD siRNA. As Fra-2, these proto-oncogenes were highly expressed in primary ATL cells but not in normal CD4+ T cells. Collectively, aberrantly expressed Fra-2 in association with JunD may play a major role in CCR4 expression and oncogenesis in ATL.
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Keywords: adult T-cell leukemia; CCR4; Fra-2; JunD; c-Myb; MDM2; BCL-6

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Introduction

Adult T-cell leukemia (ATL) is a highly aggressive malignancy of mature CD4+CD25+ T cells etiologically associated with human T-cell leukemia virus type 1 (HTLV-1; Yamamoto and Hinuma, 1985). HTLV-1 encodes a potent viral transactivator Tax that activates the HTLV-1 long terminal repeat (LTR) and also induces the expression of various cellular target genes, including those encoding cytokines, cytokine receptors, chemokines, cell adhesion molecules and nuclear transcriptional factors, collectively leading to the strong promotion of cell proliferation (Yoshida, 2001; Grassmann *et al.*, 2005). However, ATL develops after a long period of latency, usually several decades, during which oncogenic progression is considered to occur through the accumulation of multiple genetic and epigenetic changes (Matsuoka, 2003). Furthermore, circulating ATL cells usually do not express Tax and are considered to be independent of Tax (Matsuoka, 2003). Previously, Mori *et al.* have demonstrated the strong constitutive activation of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) in primary ATL cells (Mori *et al.*, 1999, 2000). However, the molecular mechanisms of ATL oncogenesis still remain largely unknown.

CCR4 is a chemokine receptor known to be selectively expressed by Th2 cells, regulatory T cells (Treg) and skin-homing effector/memory T cells (Imai *et al.*, 1999; Iellem *et al.*, 2001; Yoshie *et al.*, 2001). Previously, we and others showed that ATL cells in the majority of cases are strongly positive for surface CCR4 (Yoshie *et al.*, 2002; Ishida *et al.*, 2003; Nagakubo *et al.*, 2007). Ishida *et al.* have also demonstrated a significant correlation of CCR4 expression with skin involvement and poor prognosis in ATL patients (Ishida *et al.*, 2003). Furthermore, several groups have reported that FOXP3, a forkhead/winged helix transcription factor and a specific marker of Treg (Hori *et al.*, 2003), is frequently expressed in ATL (Karube *et al.*, 2004; Matsubara *et al.*, 2005), supporting the notion that at least a fraction of ATL cases are derived from Treg.

It is also notable that primary ATL cells express CCR4 at levels much higher than normal resting CD4+CD25+ T cells (Nagakubo *et al.*, 2007). Given

that CCR4 is not inducible by Tax (Yoshie *et al.*, 2002), transcription factor(s) constitutively active in ATL cells may be responsible for CCR4 expression. Here, we demonstrate that Fra-2, one of the AP-1 family members (Shaulian and Karin, 2002; Eferl and Wagner, 2003), is aberrantly expressed in primary ATL cells. We further demonstrate that the Fra-2/JunD heterodimer plays a major role in both CCR4 expression and cell proliferation in ATL cells. Furthermore, we demonstrate that the proto-oncogenes c-Myb, BCL-6 and MDM2 (Oh and Reddy, 1999; Pasqualucci *et al.*, 2003; Vargas *et al.*, 2003) are the downstream target genes of the Fra-2/JunD heterodimer and are highly expressed in primary ATL cells. Thus, aberrantly expressed Fra-2 in association with JunD may be involved in ATL oncogenesis.

Results

Analysis of CCR4 promoter activity in ATL-derived cell lines

To examine the transcriptional regulation of CCR4 expression in ATL, we constructed a reporter plasmid carrying the CCR4 promoter region from −983 to +25 bp (the major transcriptional initiation site, +1) fused with the luciferase reporter gene. As shown in Figure 1a, pGL3-CCR4 (−983/+25) showed much stronger promoter activities in ATL cell lines (HUT102 and ST1) than in control human T-cell lines (MOLT-4 and Jurkat). We therefore generated a series of 5′-truncated promoter plasmids and examined their activity in ATL cell lines. As shown in Figure 1b, the promoter region from −151 to −96 bp was the major positive regulatory region in both cell lines. The TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html) revealed various potential transcriptional elements in this region (Figure 1c). To identify the actual regulatory elements, we introduced a mutation in each potential element and examined the promoter activity in ATL cell lines. As shown in Figure 1d, a mutation at the AP-1 site or the GATA-3 site significantly reduced the promoter activity. Moreover, double mutations targeting both sites further reduced the promoter activity.

Constitutive expression of Fra-2, JunB and JunD in primary ATL cells

AP-1 is known to be involved in tumorigenesis (Shaulian and Karin, 2002; Eferl and Wagner, 2003), while GATA-3 regulates Th2-type gene expression (Rengarajan *et al.*, 2000). Therefore, we focused on AP-1 in the subsequent study. AP-1 constitutes a heterodimer of a member of the Fos family (c-Fos, FosB, Fra-1 and Fra-2) and a member of the Jun family (c-Jun, JunB and JunD) or a homodimer of the Jun family (Shaulian and Karin, 2002; Eferl and Wagner, 2003). Even though AP-1 was shown to be constitutively active in primary ATL cells (Mori *et al.*, 2000), it has not been clarified which members of AP-1 are actually

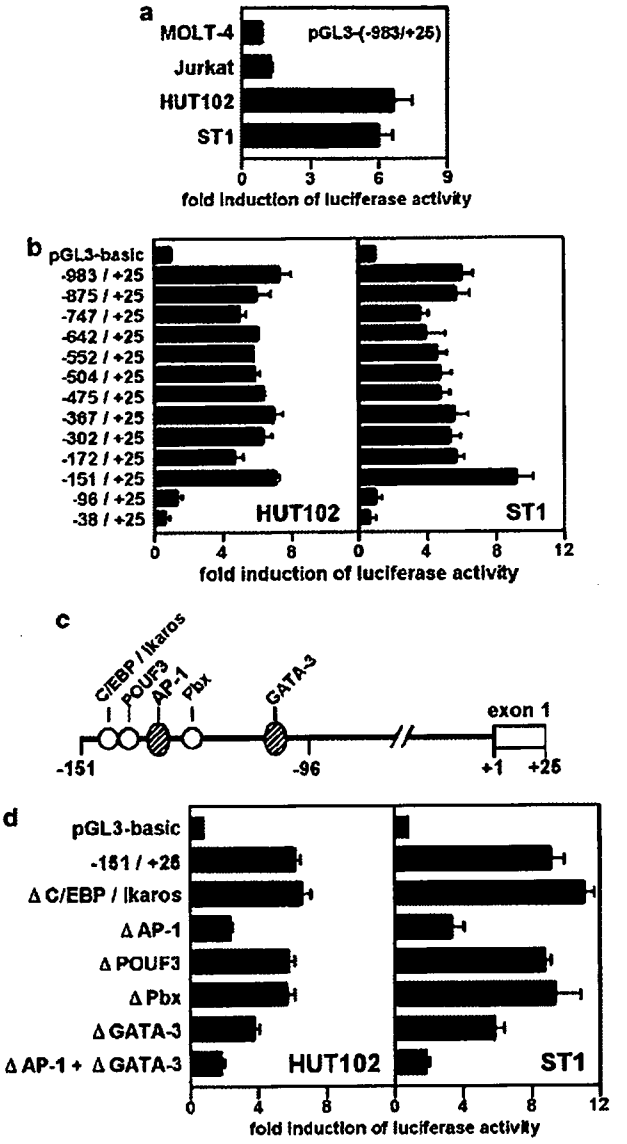

Figure 1 Identification of regulatory elements in the CCR4 promoter. Cells were transfected with pSV-β-galactosidase and pGL3-basic or pGL3-basic inserted with the CCR4 promoter regions as indicated. After 24–27 h, luciferase assays were performed. Promoter activation was expressed by the fold induction of luciferase activity in cells transfected with the CCR4 promoter–luciferase constructs versus cells transfected with the control pGL3-basic. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments. (a) Selective activation of the CCR4 promoter in adult T-cell leukemia (ATL) cell lines. MOLT-4 and Jurkat: control human T-cell lines; HUT102 and ST1: ATL cell lines. (b) Deletion analysis. The promoter region from −151 to −96 bp is necessary and sufficient for reporter gene expression in the two ATL cell lines. (c) The schematic depiction of potential regulatory elements in the promoter region from −151 to −96 bp. (d) Mutation analysis. ΔC/EBP/Ikaros (from TCTTGGGAAA TGA to TCTTGCAAAATGA), ΔAP-1 (from AATGACTAAGA to AATGTCAAAGA), ΔPOUF3 (from CTTGGGAAATGA to CTTGGGAGGTGA), ΔPbx (from AAGAATCAT to AAGA CCCAT) and ΔGATA-3 (from TTCTATCAA to TTCTGACAA). The potential AP-1 and GATA-3 sites present within the −151 to −96 bp region are the major elements for CCR4 promoter activation in the two ATL cell lines.

expressed in primary ATL cells. We therefore first examined the mRNA expression of the AP-1 family members in primary ATL cells freshly isolated from patients in comparison with normal CD4+ T cells in resting, activated and Th1/Th2-polarized conditions (Figure 2a). As reported previously (Yoshie *et al.*, 2002; Nagakubo *et al.*, 2007), primary ATL cells consistently expressed CCR4 at levels much higher than various normal CD4+ T-cell populations, including Th2-polarized cultured T cells. Furthermore, primary ATL cells consistently expressed Fra-2 in sharp contrast to various normal CD4+ T-cell populations that were essentially negative for Fra-2 expression. Similar to various normal CD4+ T-cell populations, primary ATL

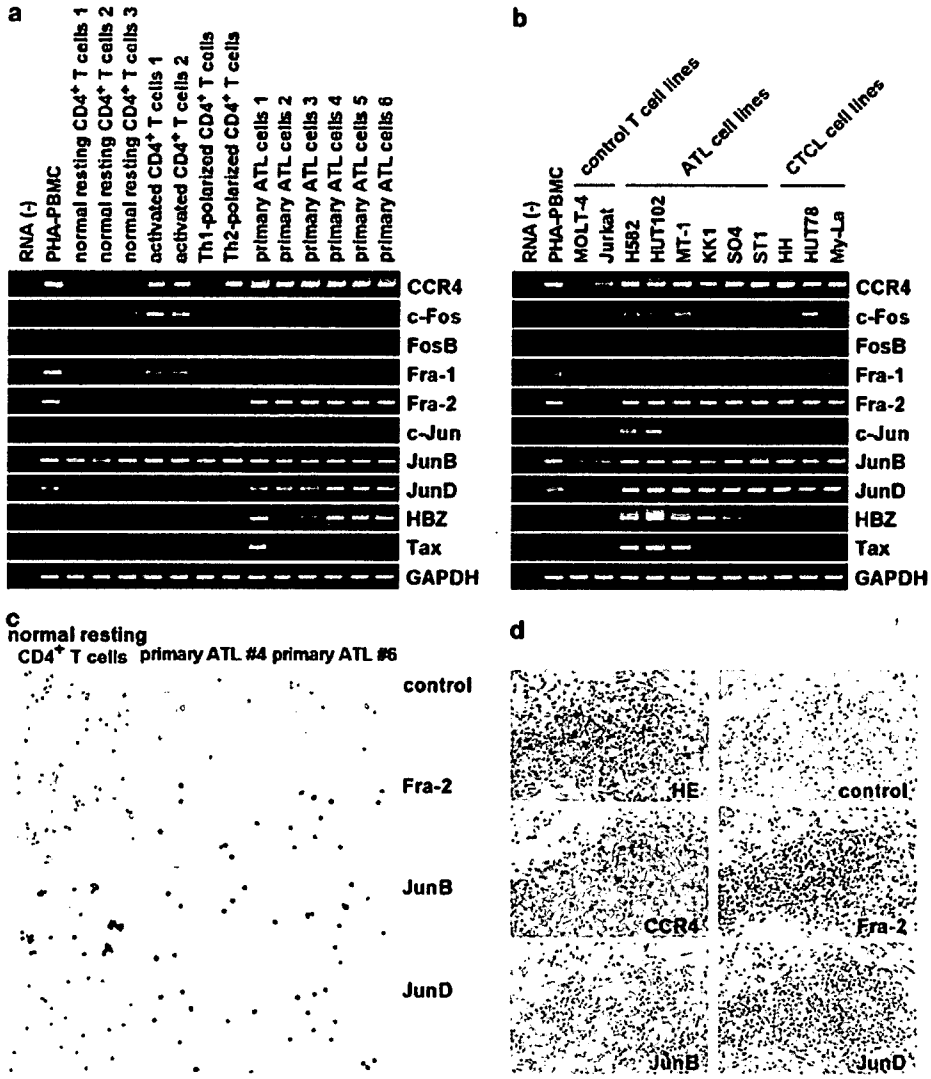

Figure 2 Constitutive expression of Fra-2, JunB and JunD in adult T-cell leukemia (ATL). (a) Reverse transcription (RT)–PCR analysis for the expression of the AP-1 family in normal T cells and primary ATL cells. Normal resting CD4+ T cells (purity, >96%) from healthy donors ($n=3$), activated CD4+ T cells from normal donors ($n=2$), Th1-polarized cultured CD4+ T cells, Th2-polarized cultured CD4+ T cells and freshly isolated primary ATL cells (>90% leukemic cells) from patients ($n=6$) were examined as indicated. Normal peripheral blood mononuclear cells treated with phytohemagglutinin (PHA-PBMC) served as a positive control. GAPDH served as a loading control. The representative results from at least two separate experiments are shown. (b) RT–PCR analysis for the expression of the AP-1 family in human T-cell lines. Two control human T-cell lines, six ATL cell lines and three CTCL cell lines were examined as indicated. PHA-PBMC served as a positive control. GAPDH served as a loading control. The representative results from two separate experiments are shown. (c) Immunocytochemical staining for Fra-2, JunB and JunD in normal CD4+ T cells and primary ATL cells. Normal CD4+ T cells from healthy donors (purity, >96%) and primary ATL cells (leukemic cells, >90%) from two patients were stained with anti-Fra-2, anti-JunB or anti-JunD. Normal rabbit IgG was used as the negative control (control). The representative results from two separate experiments are shown. Original magnification: × 400. (d) Immunohistochemical staining of CCR4, Fra-2, JunB and JunD in ATL skin lesions. Tissue sections from ATL skin lesions ($n=6$) were stained with anti-CCR4, anti-Fra-2, anti-JunB or anti-JunD. Mouse IgG_1 and normal rabbit IgG were used as the negative controls (control). Tissue sections were counterstained using Gill's hematoxylin. The representative results from a single donor are shown. Original magnification: × 400.

cells also constitutively expressed JunD and JunB even though JunD expression appeared to be upregulated in primary ATL cells. Other members of the AP-1 family were mostly negative in primary ATL cells, while activated normal CD4$^+$ T cells expressed c-Fos, Fra-1 and c-Jun at high levels. There was no correlation in expression between Fra-2 and the virally encoded HTLV-1 basic leucine zipper factor HBZ or Tax in primary ATL cells. We also confirmed that Fra-2 is not inducible by Tax using JPX-9, a subline of Jurkat carrying the HTLV-1 Tax gene under the control of the metallothionein gene promoter (Nagata *et al.*, 1989; data not shown). Thus, the constitutive expression of Fra-2 is highly unique for primary ATL cells.

We also examined expression of the same set of genes in various human T-cell lines. As shown in Figure 2b, compared to control T-cell lines, ATL cell lines consistently expressed CCR4 and Fra-2 at high levels. ATL cell lines also expressed JunB and JunD at high levels. HTLV-1 Tax has been shown to induce various AP-1 family members (Nagata *et al.*, 1989; Iwai *et al.*, 2001), which may be involved in HTLV-1 gene expression and cell proliferation (Jeang *et al.*, 1991). Consistently, ATL cell lines expressing Tax (H582, HUT102 and MT-1) also expressed other AP-1 family members at low levels. Cutaneous T-cell lymphomas (CTCLs) are a subset of HTLV-1-negative T-cell lymphomas resembling ATL and known to be frequently positive for CCR4 (Kim *et al.*, 2005). CTCL cell lines were also found to strongly express CCR4, Fra-2, JunB and JunD. Thus, the constitutive expressions of Fra-2, JunB and JunD were shared by CCR4-expressing ATL and CTCL cell lines.

We also examined the Fra-2, JunB and JunD protein expression in freshly isolated primary ATL cells and normal resting CD4$^+$ T cells. As shown in Figure 2c, primary ATL cells were indeed stained strongly positive for Fra-2, while normal CD4$^+$ T cells were totally negative for Fra-2. Primary ATL cells were also strongly positive for JunB and JunD, while normal CD4$^+$ T cells were variably positive for JunB and JunD at the single cell level. These results were highly consistent with the results from reverse transcription (RT)–PCR; Figure 2a). We also confirmed the CCR4, Fra-2, JunB and JunD protein expression in skin-infiltrating ATL cells (Figure 2d).

Activation of the CCR4 promoter by Fra-2/JunB and Fra-2/JunD heterodimers

AP-1 is known to function as a heterodimer of a member of the Fos family (c-Fos, FosB, Fra-1 and Fra-2) and a member of the Jun family (c-Jun, JunB and JunD) or a homodimer of the Jun family (Shaulian and Karin, 2002; Eferl and Wagner, 2003). We, therefore, next examined the activation of the CCR4 promoter by individual AP-1 family members singly or in combination. As recipients, we used two T-cell lines, namely, MOLT-4 and Jurkat. The expression levels of AP-1 members, including Fra-2, JunB and JunD, were very low in these cell lines (Figure 2b). As shown in Figure 3a, only Fra-2/JunB or Fra-2/JunD potently activated the CCR4 promoter in both cell lines. We confirmed that other members of the AP-1 family (c-Fos, FosB, Fra-1 and c-Jun) were transcriptionally active by using a synthetic promoter containing two tandem AP-1 consensus-binding sites (pGL3-2xAP-1; Figure 3b). Thus, among the AP-1 family members, only the Fra-2/JunB and Fra-2/JunD heterodimers are uniquely capable of activating the CCR4 promoter. This is highly consistent with their constitutive expression in primary ATL cells (Figure 2a).

Recently, the mRNA of HTLV-1 HBZ has been shown to be expressed in primary ATL cells (Satou *et al.*, 2006). We indeed observed the expression of HBZ in some primary ATL samples (Figure 2a). HBZ has been shown to activate JunB homodimer- or JunD homodimer-dependent transcription (Basbous *et al.*, 2003; Thebault *et al.*, 2004). Therefore, we also examined the effects of HBZ as well as Tax on the CCR4 promoter in MOLT-4 and Jurkat cells. As shown in Figure 3c, HBZ alone or in combination with Fra-2, JunB, JunD, Fra-2/JunB or Fra-2/JunD showed no effect on the activation of the CCR4 promoter. Similarly, Tax had no significant effect on the CCR4 promoter either alone or in combination with Fra-2, JunB, JunD, Fra-2/JunB or Fra-2/JunD. Thus, HTLV-1 encoded HBZ or Tax neither activates the CCR4 promoter nor affects its activation by Fra-2/JunB or Fra-2/JunD.

We have also confirmed that GATA-3 is constitutively expressed in primary ATL cells and activates the CCR4 promoter (data not shown). In normal CD4$^+$ T cells, GATA-3 may be responsible for the selective expression of CCR4 in Th2 cells (Imai *et al.*, 1999; Rengarajan *et al.*, 2000).

Specific binding of Fra-2, JunB and JunD to the AP-1 site in the CCR4 promoter

We next examined the specific binding of AP-1 family members to the AP-1 site in the CCR4 promoter using the NoShift transcription factor assay, an enzyme-linked immunosorbent assay (ELISA)-like colorimetric assay that is an alternative to the electrophoretic mobility shift assay. As shown in Figure 4a, when the nuclear extracts of two control T-cell lines (MOLT-4 and Jurkat) were used, the specific binding of any AP-1 family members to the AP-1 site of the CCR4 promoter was hardly observed. On the other hand, when the nuclear extracts of two ATL cell lines (HUT102 and ST1) were used, we detected a high level of specific binding of Fra-2, JunB and JunD to the AP-1 site. These results are highly consistent with the results from RT–PCR analyses (Figure 2b) and the luciferase reporter assays (Figure 3a).

By using the chromatin immunoprecipitation (ChIP) assay, we further examined the binding of Fra-2, JunB and JunD to the AP-1 site of the CCR4 promoter *in vivo*. As shown in Figure 4b, we detected specific binding of Fra-2, JunB and JunD to the AP-1 site of the endogenous CCR4 promoter in primary ATL cells but not in normal CD4$^+$ T cells. These results further

support the hypothesis that the CCR4 gene is a direct target gene of Fra-2/JunB and Fra-2/JunD heterodimers in primary ATL cells.

Effects of Fra-2, JunB and JunD small interfering RNAs on CCR4 expression and cell proliferation

To examine the role of Fra-2, JunB and JunD in CCR4 expression and cell proliferation in ATL cells, we next employed the small interfering RNA (siRNA) knockdown technique. As shown in Figure 5a, Fra-2 siRNA, JunB siRNA and JunD siRNA specifically reduced Fra-2 mRNA, JunB mRNA and JunD mRNA, respectively, in two ATL cell lines. On the other hand, control siRNA showed no such effect. Under these conditions, we examined the effects of these siRNAs on CCR4 expression and cell growth. As shown in Figure 5b, Fra-2 siRNA and JunD siRNA reduced CCR4 expression by approximately 50% in both cell lines, whereas JunB siRNA had hardly any inhibitory effect and control siRNA showed no inhibitory effect. Furthermore, as shown in Figure 5c, Fra-2 siRNA and JunD siRNA significantly reduced cell proliferation in both cell lines, whereas JunB siRNA or control siRNA did not. None of the siRNAs affected the growth of the control T-cell lines MOLT-4 and Jurkat. We also compared the effects of single and double knockdown of Fra-2 and JunD on cell growth in two ATL cell lines (Figure 5d). Compared to the effect of single knockdown of Fra-2 or JunD, no additive effect was observed by double knockdown of Fra-2 and JunD in both cell lines. These results may be consistent with the notion that Fra-2 and JunD promote growth in ATL cell lines by functioning as a heterodimer.

To further demonstrate the growth-promoting effects of Fra-2 and JunD, we performed stable transfection of Fra-2 and JunD in the control T-cell line Jurkat. As shown in Figure 5e, Jurkat cells overexpressing Fra-2 or JunD (see inset) indeed showed enhanced growth compared to those transfected with the vector alone. We were, however, unable to isolate Fra-2/JunD double transfectants in Jurkat, probably because of some adverse effects on Jurkat cells by the overexpression of both Fra-2 and JunD.

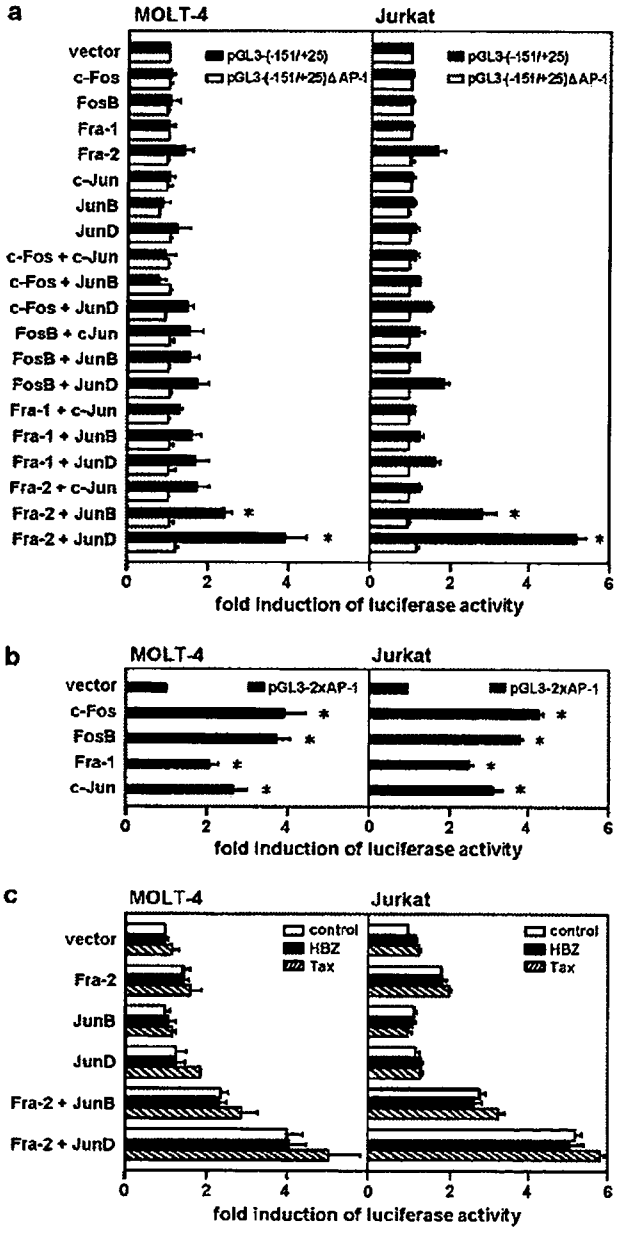

Figure 3 Transactivation of the CCR4 promoter by Fra-2/JunD and Fra-2/JunB. (a) Transactivation of the CCR4 promoter with or without the AP-1 site. MOLT-4 and Jurkat cells were cotransfected with pSV-β-galactosidase and pGL3-CCR4 (−151/+25) or pGL3-CCR4 (−151/+25)ΔAP-1 and an expression vector for c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, JunD or a control vector as indicated. After 24–27 h, luciferase assays were performed in triplicate. Promoter activation was expressed as the fold induction of luciferase activity in cells transfected with an indicated AP-1 expression vector versus cells transfected with the vector alone. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments. $^*P<0.05$. (b) Transactivation of a synthetic promoter with two copies of the consensus AP-1 site. MOLT-4 and Jurkat cells were cotransfected with pSV-β-galactosidase and pGL3-2xAP-1 and an expression vector for c-Fos, FosB, Fra-1, c-Jun or the vector alone as indicated. Promoter activation was expressed as the fold induction of luciferase activity in cells transfected with an indicated expression vector versus cells transfected with a control vector. After 24–27 h, luciferase assays were performed in triplicate. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments. $^*P<0.05$. (c) Effect of HBZ or Tax on the activation of the CCR4 promoter. MOLT-4 and Jurkat cells were cotransfected with pSV-β-galactosidase and the pGL3-basic vector or pGL3-CCR4 (−151/+25) and an expression vector for Fra-2, JunB, JunD or a control vector and an expression vector for HBZ, Tax or a control vector as indicated. After 24–27 h, luciferase assays were performed in triplicate. Promoter activation was expressed as the fold induction of luciferase activity in cells transfected with an indicated expression vector versus cells transfected with a control vector. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments.