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Figure Legend

Figure 1. Efficacy of anti-tumor necrosis factor 3 months after the beginning of treatment.

A, Response rate of HTLV-1 positive (n= 10) and negative patients (n= 20) with rheumatoid arthritis (RA) according to European League Against Rheumatism (EULAR) improvement criteria.

B, Disease activity of HTLV-1 positive (n= 10) and negative (n= 20) patients with RA according to EULAR improvement criteria.

C, Changes in the levels of C-reactive protein, erythrocyte sedimentation rate and disease activity score in 28 joints at 3 months (3M) after anti-TNF therapy.

HDA: high disease activity, MDA: moderate disease activity, LDA: low disease activity, REM: remission. HTLV-1: human T- lymphotropic virus type 1.

Figure 2. Continuation rate of anti-tumor necrosis factor therapy in human T- lymphotropic virus type 1 positive (n= 10) and negative (n= 20) patients with rheumatoid arthritis.

HTLV-1: human T-cell lymphotropic virus type 1.

	HTLV-1 positive (N= 10)	HTLV-1 negative (N=20)	P-value †
No.	10	20	-
Age, median (IQR) years	70.0 (8.5)	68.5 (11.7)	0.98
Duration, median (IQR) years	5.0 (5.0)	9.0 (19.5)	0.21
Disease activity markers			
CRP, median (IQR) mg/dl	4.1 (4.2)	0.7 (1.3)	0.0003
ESR, median (IQR) mm/60min	74.5 (37.5)	65.0 (34.5)	0.15
TJC28, median (IQR)	4.5 (4.3)	4.5 (4.0)	0.72
SJC28, median (IQR)	4.5 (3.8)	2.0 (5.0)	0.35
DAS28, median (IQR)	5.8 (0.8)	5.2 (0.8)	0.18
Disease activity according to EULAR criteria			
High disease activity (DAS28: >5.1) [%]	80	70	-
Moderate disease activity (DAS28: 3.2 - 5.1) [%]	20	25	-
Low disease activity (DAS28: <3.2) [%]	0	5	-
Serological markers			
RF positive [%]	80	80	> 0.99
ACPA positive [%]	90	100	0.33
Treatment			
DMARDs (%)	33	45	> 0.99
Methotrexate median mg/week (IQR) [%]	10 (2.0) [50]	8.0 (2.5) [75]	0.23
Predonisolon median mg/day (IQR) [%]	5.5 (2.0) [90]	3.5 (3.0) [70]	0.37

Baseline characteristics of patients.
HTLV-1 = human T-lymphotrophic virus type 1; IQR = interquartile range; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; TJC28 = tender joint counts in 28 joints; SJC28 = swollen joint counts in 28 joints; DAS28 = disease activity score in 28 joints; RF= rheumatoid factor; ACPA = anti-citrullinated protein antibody; DMARDs = disease-modifying antirheumatic drugs
† By Mann-Whiteny U test, Fisher's exact test

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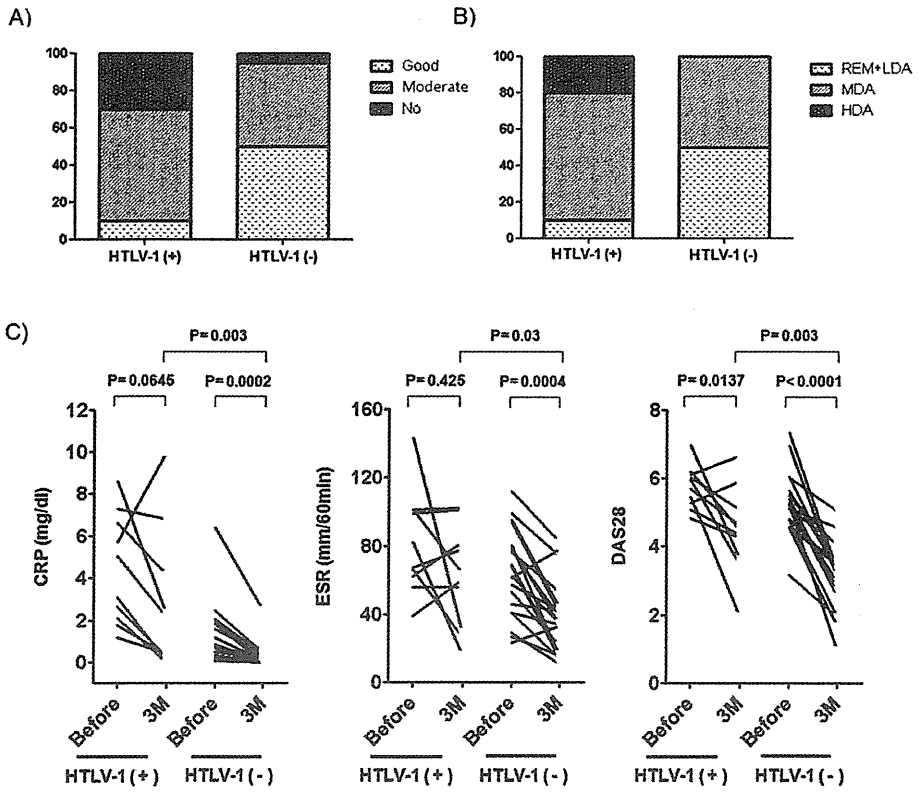

Efficacy of anti-tumor necrosis factor 3 months after the beginning of treatment.
A, Response rate of HTLV-1 positive (n= 10) and negative patients (n= 20) with rheumatoid arthritis (RA) according to European League Against Rheumatism (EULAR) improvement criteria.
B, Disease activity of HTLV-1 positive (n= 10) and negative (n= 20) patients with RA according to EULAR improvement criteria.
C, Changes in the levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and disease activity score in 28 joints (DAS28) at 3 months (3M) after anti-TNF therapy.
HDA: high disease activity, MDA: moderate disease activity, LDA: low disease activity, REM: remission.
HTLV-1: human T- lymphotropic virus type 1.

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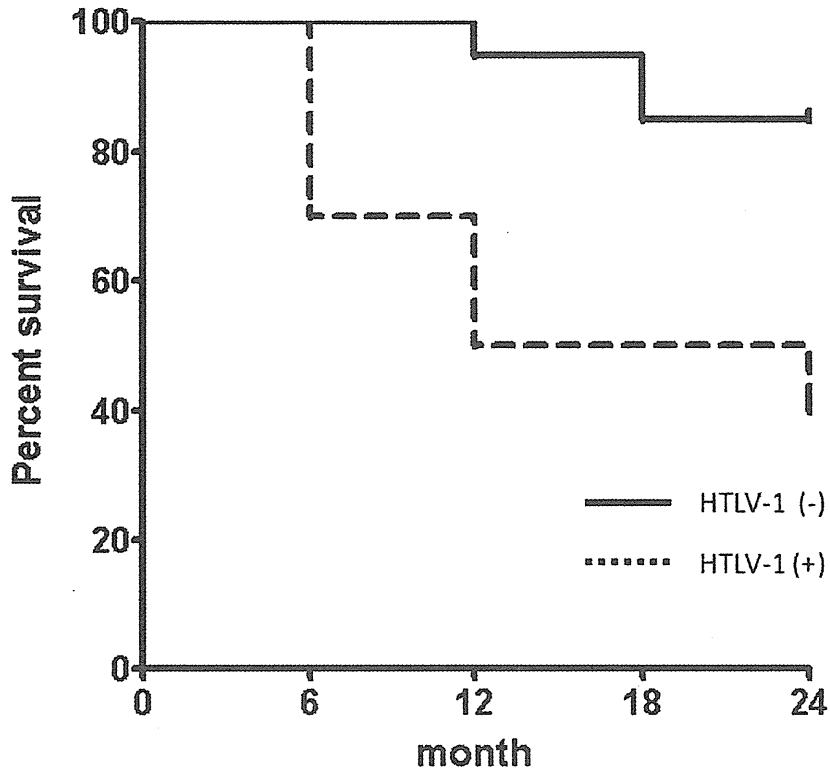

Continuation rate of anti-tumor necrosis factor therapy in human T- lymphotropic virus type 1 positive (n= 10) and negative (n= 20) patients with rheumatoid arthritis.
HTLV-1: human T-cell lymphotropic virus type 1.

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HTLV-I 母子感染対策

齋藤 滋*

HTLV-I は，ATL や HAM の原因ウイルスであるが，いったん感染すると薬剤などでウイルスを排除できない。そのため，感染予防が極めて重要であり，母乳を介した母子感染を減少させるため，妊婦にスクリーニングが行われている。一次スクリーニングで陽性となれば，必ずウェスタンブロット(WB)法を確認検査として行い，陽性となればキャリアと同定し，陰性であれば母乳哺育を勧める。判定保留者には，PCR 法を行うことを提案する。キャリアに対して，人工乳，3カ月までの短期母乳，凍結母乳の3つの方法を提示し，それぞれのメリット，デメリットを説明し，患者に選択してもらう。短期母乳，凍結母乳を選択した場合，継続的な地域の助産師，保健師のサポートが必要である。

はじめに

1981 年に HTLV-I が ATL(adult T cell leukemia：成人T細胞白血病)の原因ウイルスとして同定され[1]，1991 年に重松班より HTLV-I 母子感染に対する見解が発表され[2]，① 全国でキャリアは120万人いる，② 母子感染率は15～25%に生じる，③ 母子感染の主体は母乳を介する経路(母子感染の約 90%)である，④ 新しい差別の対象とならないため，キャリア率の高い地域以外では対策不要，と報告された。しかし，2007 年の厚生労働研究 山口班報告[3]で，献血者からの HTLV-I キャリア数を推定すると，① 全国で 108 万人いること，② HTLV-I キャリアが全国に拡散し，もはや九州，沖縄だけのウイルスでなくなったこと，が明らかとなった。HTLV-I 感染は，① 母乳を介した母子感染，② 性行為を介した感染，③ 輸血を介した感染，の3つがあるが，現在は輸血を介した感染は皆無である。ATL の発症は母乳を介した感染のみで生じること，HTLV-I キャリアの減少と ATL，HAM(HTLV-I 関連脊髄症)撲滅のためには，母子感染対策が最も効果的であることから，国は，公費で妊婦に対する HTLV-I 抗体検査を助成するようになった。このことは，HTLV-I の撲滅のためには，極めて有益であるが，突然，キャリアと判明した妊婦に対して，正しい情報を提供することが必要であるのみならず，精神的なケアやフォローアップの対応，退院後の母乳管理を含めた助産師や保健師のサポートが必要になる。これまでは任意の検査であったため，検査のメリットのみが強調されてきたが，これからはデメリットに対する十分な対応が求められる。

1．妊婦スクリーニング方法の実際

図1に示すように，妊娠 30 週までに抗体検査を行う[4]。大多数は陰性だが，一部の妊婦が陽性者となる。この際，一次抗体スクリーニングには偽陽性のあることを認識すべきであ

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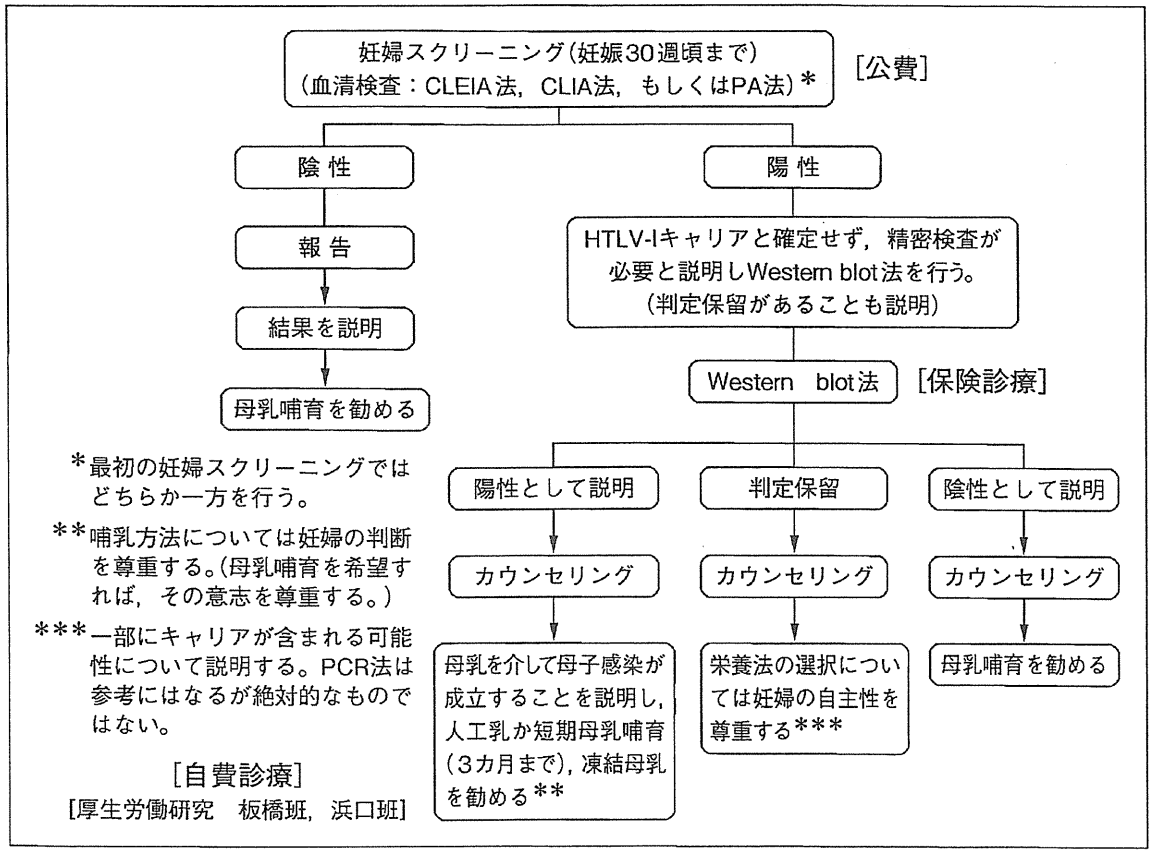

図1 HTLV-Iスクリーニングの進め方

る[4]。特にキャリアの多い九州，沖縄以外では一次抗体陽性者の20～30%程度がHTLV-Iキャリアであるにすぎない。このため，精密検査が必要と説明して，必ず確認検査であるWestern blot(WB)法を保険診療で行う。なお，この際，判定保留もあることをあらかじめ伝えておく[4]，WB法陽性者には，HTLV-Iキャリアとして説明し，HTLV-IウィルスとATL，HAMのこと，母子感染予防法があることを説明する。なお，HTLV-Iキャリアであるとの説明は，本人にのみ行う。家族への説明は本人の許可を得た上で行う。WB法陰性の場合は，キャリアでないため妊婦がATLやHAMになる心配もなく，また母乳感染もないことから，母乳哺育を勧める。20～30%に判定保留となるケースがある。この場合，厚生労働研究 板橋班[5]協力施設(http://htlv-1mc.org/参照)に協力していただければ，無償でPCR法を行うことができる。協力施設が近隣にない場合，PCR法は自費診療となる。PCR法が陽性であれば，HTLV-Iキャリアとして説明する。PCR法陰性の場合，キャリアでないか，HTLV-Iウイルス量が極めて少ないキャリアのいずれかである。PCR法が陰性であった際，積極的に母乳を制限するエビデンスはない[4]。しかし，絶対に安全という保障もない。筆者は個人的に「おそらく母乳を長期投与しても，完全人工乳での母子感染率の3%を上回らないであろう。しかし，完全に安全というエビデンスは今のところない。どうしても心配な場合は，短期母乳や凍結母乳でもよい」と説明している。このような症例が厚生労働研究 板橋班に登録され，母乳の長期投与の安全性が証明されれば，安心して母乳哺育を推奨できるようになるので[5]，ぜひともご協力いただきたい。富山県でのデータを基に全国の判定保留者数を推定すると，年間約650人程度存在することになる。

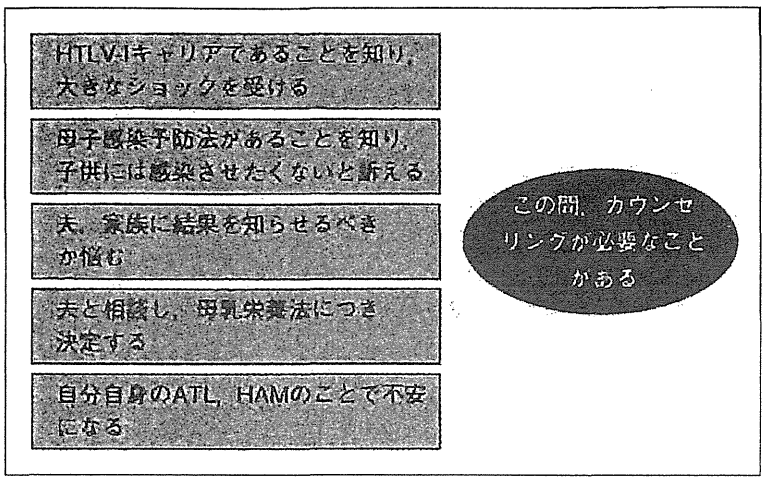

図2 典型的なキャリア例

2．HTLV-I キャリアに対する説明

1 発病リスクについて

図2に典型的なHTLV-Iキャリアの例を示す。親族にATLやHAMの方がいる場合は，比較的冷静に結果を受容できる場合が多いが，多くの場合，大きなショックを受ける。まずこのウイルスがATLやHAMの原因ウイルスであることを説明するが，ATLの生涯発症率は5%，HAMの発生率は0.25%である。HTLV-Iキャリアの会に「ATLになる確率は5%」と説明したほうがよいか，「40歳を過ぎてから年間およそ1,000人に1人(タバコによる肺癌発症の半分程度)」と説明したほうがよいかを尋ねたところ，妊娠時には精神的に不安定となるため，後者のマイルドな説明にしてほしいとの意見が多かった。説明の際の参考にしていただきたい。

2 栄養法の選択について

次に母子感染を減少させるために，①完全人工栄養，②3カ月までの短期母乳，③凍結解凍母乳の3つの方法があることを説明し(表1)，表2のように，それぞれの方法のメリット，デメリットを説明し，患者の意志で選択してもらう。医師から一方的に栄養方法を強要してはならない。表1に示すように，人工哺育はこれまでに1,500例を超える症例数があり，最も確実に母子感染を減少させることができる。さらに分娩48時間以内にカバサール® 1 mg 1回内服のみで，比較的容易に乳汁分泌を停止させることができる。デメリットとしては母子間の母乳を介した愛情形成ができないこと(完全人工栄養でも母子間愛情形成できる)，新生児の呼吸器系や消化管系の感染症のリスクが高くなること(IgAの補給がないため)，ミルク費用などである。3カ月までの短期母乳は，直接母乳も可能で妊婦の満足度も高いが，最大の欠点は途中で母乳哺育を止めることができず，長期母乳となる可能性があることである(約1/4が長期母乳となると推定する報告もある)。そのため産後2カ月末～3カ月に入った頃から，徐々に粉ミルクに切り替えることと，地域の助産師や保健師と協力して，訪問看護などを行い，母乳指導を行うことが望ましい。凍結母乳は栄養的に母乳と変らず，4カ月以上投与できるメリットがあるが，手間がかかるのが最大の欠点である。搾乳器を用いて母乳を搾乳した後に母乳

表1 HTLV-I 母子感染率

1990年	母乳哺育	103/788(13.1%)
	人工哺育	36/953(3.8%)
1990年以降	母乳哺育	
	4カ月以上	93/525(17.7%)
	3カ月以下	3/162(1.9%)
	人工哺育	51/1,553(3.3%)
	凍結母乳	2/64(3.1%)

(厚生労働研究　齋藤班)

表2 栄養法のメリット，デメリット

方法	メリット	デメリット
完全人工栄養	・最も確実に母子感染を予防する（18%→3%） ・母乳を止める方法が確立している	・完全には母子感染を予防できない ・母子間の母乳哺育を介したスキンシップ，愛情形成が行えない ・新生児，乳児期の子供の感染症のリスク（IgAが補供されないため） ・ミルク代（費用）
3カ月までの短期母乳	・直接哺乳も可能 ・母子間愛情形成に役立つ	・症例数が少なく十分には安全性が確立していない ・途中で母乳哺育を止められず，ズルズル長期母乳になる可能性あり（3カ月に入った頃から混合栄養に切り替える必要あり）
凍結解凍母乳	・栄養的には母乳と同じ ・3カ月以上投与できる	・手間がかかる ・症例数が少なく十分には安全性が確立していない

パックに入れ，日付と量を明記して家庭用冷凍庫で−20℃で24時間以上凍らせる。哺乳の際は流水または微温湯で解凍し（電子レンジを使うと有効成分が壊れてしまうので不適切），哺乳瓶で与える。この方法を選択した症例に対しても助産師や保健師の分娩後の支援が望ましい。

富山県では，HTLV-Iキャリアの方が出産し，退院する際に，地域の保健師がサポートする体制があることを説明し，希望した場合に，未熟児・ハイリスク児訪問のシステムを用いて，定期的に保健師がフォローアップしている。このフォローアップ体制は，富山県HTLV-I母子感染対策検討会で決定されたので，医師（産婦人科医，小児科医），助産師，保健師，行政が協力してフォローアップ体制を構築することができた。参考にしていただきたい。

3 夫，家族への説明について

次に出てくる質問は，夫や家族に結果を知らせるべきかで妊婦は悩む。夫婦の状況によっても異なるが，筆者は原則的にキャリア妊婦の許可を得た上で，夫に説明している。これは，①HTLV-Iは「親の意志」によって防ぐことができる感染症であり，子供の将来を決定するためには2人で責任を負うほうがよいから，②キャリアである妊婦を支えてくれるのは夫であるから，③夫に無断で人工乳，短期母乳，凍結母乳を選択すると，トラブルになる可能性が高いからである。家族に対しての説明は，医療者から提案はしないが，キャリア妊婦から依頼された際には，行うこともある。ただし，HTLV-Iが性行為により感染するため，家庭問題に発展することがあり，注意を要する。

4 妊婦の健康への配慮

夫と相談の上，哺乳方法を決定すると，妊婦は自分の健康のことが心配になってくる。ATLやHAMのことは，その概略は産婦人科医でも説明できるが，詳しい説明は血液内科医や神経内科医に依頼する。各都道府県で，HTLV-I母子感染対策協議会が設置されているので，担当の医師を決めておき，相談に応ずる体制づくりが必要である。その際，重要なことは，普通の生活を送ってよいと説明することで，定期的なフォローアップも原則必要ない。ただし，HAMの場合，徐々に進行することが多いので，歩行障害や膀胱直腸障害が出現すれば，早めに来院するように指示したほうが，病気の進行をくい止めることができる。またリンパ節腫脹，発熱，皮膚症状が出現すればATLの可能性があるので，血液内科を受診するように指示しておく。

表3 今後のHTLV-Iキャリア数とATL患者の推定数

	分娩数（人）	キャリア率	推定キャリア数（人）	母子感染例（人）	母子感染例からのATL生涯発生例（人）
2010年	107万人	0.13%	1,441人	43人	2.2人
2040年	100万人	0.02%	200人	6人	0.3人

仮定1：栄養法の介入により母子感染が3%に減少
仮定2：生涯ATL発症率を5%

この事業をあと30年続けると日本からATLを撲滅できる。

3．以前と現在の体制のどこが変化したのか

まず，国が公費で妊婦のHTLV-I抗体検査費用を補助し，日本からATL，HAMといった難病を撲滅するよう動き出したことである。第2として，以前は一方的に医師が栄養方法を決めていたが，人工乳，3カ月までの短期母乳，凍結母乳の3つの栄養方法を呈示し，メリット，デメリットを説明した上で患者の意志で，栄養方法を選択するようになった。第3として，突然，HTLV-Iキャリアと告知された妊婦の精神的サポート，母乳栄養法の具体的なサポートを医師，助産師，地域の保健師で協力して行うように，都道府県にHTLV-I母子感染対策協議会や相談窓口が設置されたことである。つまり，検査するだけでなく，キャリアの方々の精神的なサポート，母乳管理の具体的なサポートを行いながら，HTLV-I母子感染を予防する体制が作られたことになる。

おわりに

HTLV-I母子感染を防止するのみならず，キャリアの心のケアも配慮した国を挙げての体制ができあがった。表3に示すように，栄養法の介入により母子感染率を3%に減少させるため，この事業をあと30年続けると，日本からATLを撲滅できる。全国で妊婦のHTLV-Iスクリーニングが正しく行われ，HTLV-I母子感染が減少し，かつキャリアの健康が維持されることを切望する。

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5) 厚生労働科学研究費補助金 成育疾患克服等次世代育成基盤研究事業 HTLV-I母子感染予防に関する研究―HTLV-I抗体陽性妊婦からの出生児のコホート研究 平成23年度総括・分担研究報告書 研究代表者 板橋家頭夫.

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The CD3 versus CD7 Plot in Multicolor Flow Cytometry Reflects Progression of Disease Stage in Patients Infected with HTLV-I

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Abstract

Purpose: In a recent study to purify adult T-cell leukemia-lymphoma (ATL) cells from acute-type patients by flow cytometry, three subpopulations were observed in a CD3 versus CD7 plot (H: $CD3^{high}CD7^{high}$; D: $CD3^{dim}CD7^{dim}$; L: $CD3^{dim}CD7^{low}$). The majority of leukemia cells were enriched in the L subpopulation and the same clone was included in the D and L subpopulations, suggesting clonal evolution. In this study, we analyzed patients with indolent-type ATL and human T-cell leukemia virus type I (HTLV-I) asymptomatic carriers (ACs) to see whether the CD3 versus CD7 profile reflected progression in the properties of HTLV-I-infected cells.

Experimental Design: Using peripheral blood mononuclear cells from patient samples, we performed multi-color flow cytometry. Cells that underwent fluorescence-activated cell sorting were subjected to molecular analyses, including inverse long PCR.

Results: In the D(%) versus L(%) plot, patient data could largely be categorized into three groups (Group 1: AC; Group 2: smoldering- and chronic-type ATL; and Group 3: acute-type ATL). Some exceptions, however, were noted (*e.g.*, ACs in Group 2). In the follow-up of some patients, clinical disease progression correlated well with the CD3 versus CD7 profile. In clonality analysis, we clearly detected a major clone in the D and L subpopulations in ATL cases and, intriguingly, in some ACs in Group 2.

Conclusion: We propose that the CD3 versus CD7 plot reflects progression of disease stage in patients infected with HTLV-I. The CD3 versus CD7 profile will be a new indicator, along with high proviral load, for HTLV-I ACs in forecasting disease progression.

Citation: Kobayashi S, Tian Y, Ohno N, Yuji K, Ishigaki T, et al. (2013) The CD3 versus CD7 Plot in Multicolor Flow Cytometry Reflects Progression of Disease Stage in Patients Infected with HTLV-I. PLoS ONE 8(1): e53728. doi:10.1371/journal.pone.0053728

Editor: Jean-Pierre Vartanian, Institut Pasteur, France

Received August 31, 2012; **Accepted** December 4, 2012; **Published** January 22, 2013

Funding: This study was supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Human T-cell leukemia virus type I (HTLV-I) is the agent that causes HTLV-I-associated diseases, such as adult T-cell leukemia-lymphoma (ATL), HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and HTLV-I uveitis (HU) [1–3]. Approximately 10–20 million people are infected with the HTLV-I virus worldwide [4]. The lifetime risk of developing ATL is estimated to be approximately 2.5–5% [5,6]. ATL includes a spectrum of diseases that are referred to as smoldering-, chronic-, lymphoma-, and acute-type [7,8]. The chronic and smoldering types of ATL are considered indolent and are usually managed with watchful waiting until the disease progresses to aggressive (lymphoma- or acute-type) ATL [9]. Because the prognosis of ATL is poor with current treatment strategies, factors to forecast progression to ATL from asymptomatic carriers (ACs) have been researched [10–13] in the hope that they will be useful for preventive therapy under development in the early malignant stage.

Various cellular dysfunctions induced by viral genes (*e.g.*, tax and HBZ), genetic and epigenetic alterations, and the host immune system are considered to cooperatively contribute to leukemogenesis in ATL [14–16]. However, the complex mechanism may hinder determination of a clear mechanism of the pathology and make discovery of risk factors difficult. In a prospective nationwide study in Japan, high proviral load (VL,

Table 1. Clinical profile of patients infected with HTLV-I and normal controls.

Clinical sutype	Number of cases	Male	Female	Age (range)	WBC(/μl) (range)	Lymphocytes(%) (range)	Abnormal lymphocytes(%) (range)
HTLV-1 AC	40	12	28	49.9 (28–70)	5525 (2680–10360)	35.9 (22.4–59.5)	0.9 (0.0–4.4)
Smoldering	7	4	3	55.3 (43–77)	5944 (3680–8710)	32.5 (13.4–47.5)	5.8 (0.7–16.5)
Chronic	7	4	3	52.7 (37–60)	9180 (4070–12790)	45.8 (35.0–61.5)	9.2 (3.4–12.7)
Acute	13	4	9	58.8 (42–74)	15328 (4450–41480)	16.3 (1.7–50.5)	40.3 (3.0–89.6)
Normal controls	10	6	4	47.4 (27–66)	ND	ND	ND

WBC: white blood cells (normal range, 3500–9100/μl).
AC: asymptomatic carrier.
ND: analysis were not performed.
Average of age, WBC, lymphocytes (%) and abnormal lymphocytes (%) are shown.
The proportion of abonormal lymphocytes in peripheral blood WBCs was evaluated by morphological exmanination.
doi:10.1371/journal.pone.0053728.t001

over 4.17 copies/100 peripheral blood mononuclear cells) was found to be a major risk factor for HTLV-I AC developing into ATL [13]. Although VL indicates the proportion of HTLV-I-infected cells, it does not indicate size or degree of malignant progression in each clone; *i.e.*, it does not directly indicate progression of disease stage in HTLV-I infection. Moreover, the majority of ACs with high VL remained intact during the study period, indicating that a more accurate indicator of progression is needed.

In our recent study to purify monoclonal ATL cells from acute-type patients by flow cytometry, three subpopulations were observed in a CD3 versus CD7 plot of $CD4^+$ cells (H: $CD3^{high}CD7^{high}$, D: $CD3^{dim}CD7^{dim}$, L: $CD3^{dim}CD7^{low}$), and the majority of ATL cells were enriched in the L subpopulation [17]. Clonality analyses revealed that the D and L subpopulations contained the same clone, suggesting clonal evolution of HTLV-I-infected cells to ATL cells. From these findings, we speculated that the CD3 versus CD7 profile may reflect disease progression in HTLV-I infection. In this study, the CD3 versus CD7 profile by flow cytometry, combined with molecular (clonality and proviral load) characterizations, were analyzed in patients with various clinical subtypes (HTLV-I AC, and indolent and aggressive ATL). We found that the CD3 versus CD7 profile reflected disease progression of HTLV-I-infected cells to ATL cells. We also discuss the significance of this analysis as a novel risk indicator for HTLV-I ACs in forecasting progression to ATL.

Materials and Methods

Cell lines and patient samples

TL-Om1, an HTLV-I-infected cell line, established Dr. Hinuma's laboratory [18], was provided by Dr. Toshiki Watanabe (The University of Tokyo, Tokyo, Japan) and was cultured in RPMI-1640 medium containing 10% fetal bovine serum. Peripheral blood samples were collected from inpatients and outpatients at our hospital from August 2009 to November 2011. All patients with ATL were categorized according to Shimoyama's criteria [7,8]. Patients with various complications, such as autoimmune disorder and systemic infections, were excluded. Lymphoma-type patients were excluded because ATL cells are not considered to exist in peripheral blood of this clinical subtype. In patients with ATL receiving chemotherapy, blood samples were collected before treatment or during the recovery phase between chemotherapy sessions. Samples collected from 10 healthy volunteers (mean age: 47.4 years; range: 27–66 years) were used as normal controls.

The present study was approved by the research ethics committee of the institute of medical science, the university of Tokyo. Subjects provided written informed consent.

Flow cytometry and cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-treated whole blood by density gradient centrifugation, as described previously [17]. Cells were stained using a combination of phycoerythrin (PE)-CD7, APC-Cy7-CD3, Pacific Blue-CD4, and Pacific Orange-CD14. Pacific Orange-CD14 was purchased from Caltag-Invitrogen (Carlsbad, CA). All other antibodies were obtained from BD BioSciences (San Jose, CA). Propidium iodide (PI; Sigma, St. Louis, MO) was added to the samples to stain dead cells immediately prior to flow cytometry. A BD FACS Aria instrument (BD Immunocytometry Systems, San Jose, CA) was used for all multicolor flow cytometry and cell sorting. Data were analyzed using the FlowJo software (Treestar, San Carlos, CA).

Quantification of HTLV-I proviral load by real-time quantitative polymerase chain reaction (PCR)

The HTLV-I proviral load in FACS-sorted PBMCs was quantified by real-time quantitative polymerase chain reaction (PCR; TaqMan method) using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as described previously [13,17]. Briefly, 50 ng of genomic DNA was extracted from human PBMCs using a QIAamp DNA blood Micro kit (Qiagen, Hilden, Germany). Triplicate samples of the DNA were amplified. Each PCR mixture, containing an HTLV-I pX region-specific primer pair at 0.1 μM (forward primer 5′-CGGATACCCAGTCTACGTGTT-3′ and reverse primer 5′-

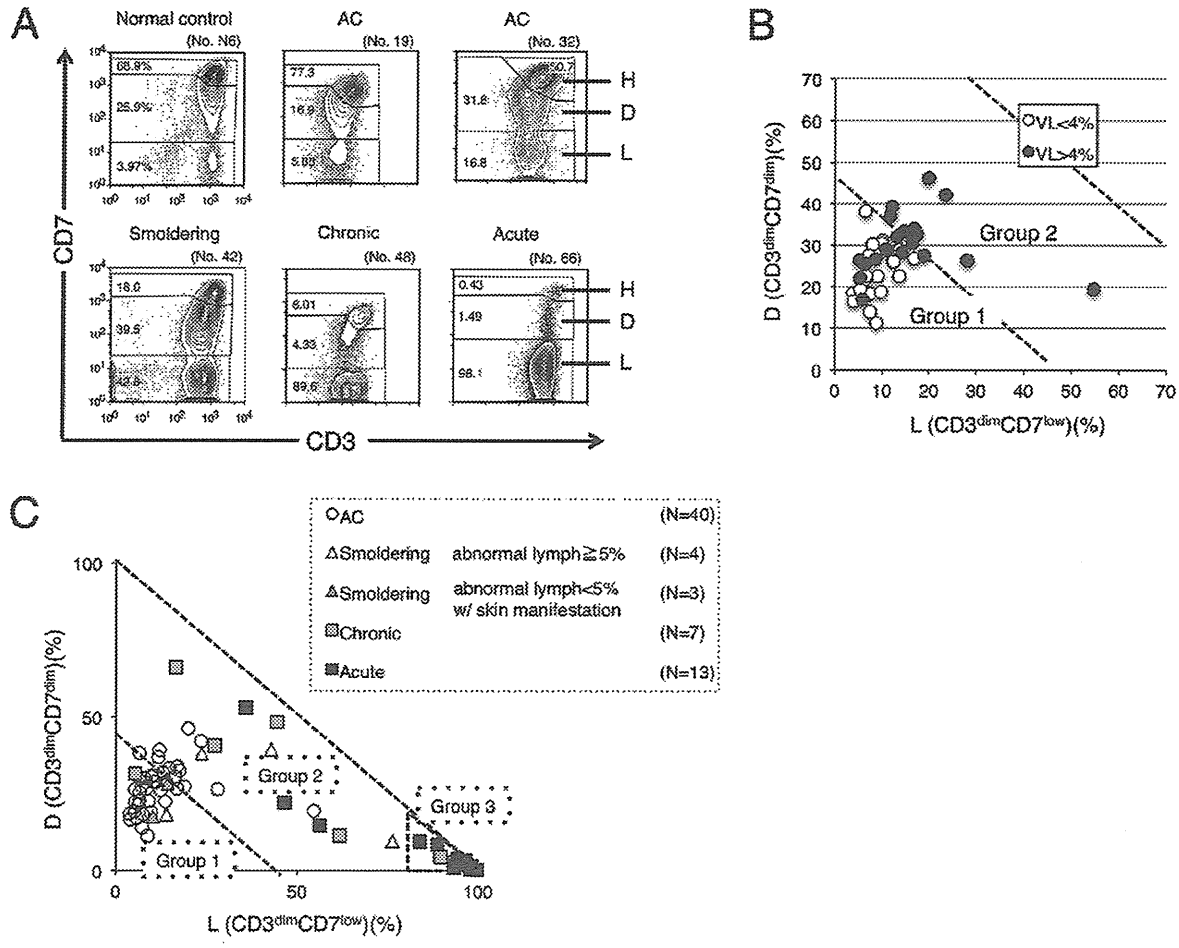

Figure 1. CD3 versus CD7 plots in flow cytometric analysis of patients who are asymptomatic HTLV-I carriers (ACs) and have various clinical subtypes of adult T-cell leukemia-lymphoma (ATL) suggest disease progression in HTLV-I infection. (A) Flow cytometric profile of an AC, various clinical subtypes of ATL (smoldering, chronic, and acute), and a normal control. Representative cases of CD3 versus CD7 plots in $CD4^+$ cells are shown. (B) A two-dimensional plot of AC cases showing the percentage of the D and L subpopulations by flow cytometry. AC cases were divided into two groups according to HTLV-I VL (greater or less than 4%). The border line (45% of D+L subpopulations) between Group 1 and 2 was set based on proviral load (VL). All AC cases with less than 4% VL were included in Group 1. All AC cases included in Group 2 had greater than 4% VL. VL<4%: n = 21; VL>4%: n = 19. All VL data in this figure were provided from the database of the Joint Study on Predisposing Factors of ATL Development (JSPFAD). (C) A two-dimensional plot of all patients showing the percentage of the D and L subpopulations. The smoldering type was divided into two categories: smoldering type with greater than 5% abnormal lymphocytes and smoldering type with less than 5% abnormal lymphocytes with skin manifestation. The two diagonal dotted lines indicate 45% and 100% of D+L subpopulations (*i.e.*, 55% and 0% of the H subpopulation). Data were categorized into three groups.
doi:10.1371/journal.pone.0053728.g001

CAGTAGGGCGTGACGATGTA-3′), FAM-labeled probe at 0.1 μM (5′- CTGTGTACAAGGCGACTGGTGCC-3′), and 1× TaqMan Universal PCR master mix (Applied Biosystems), was subjected to 50 cycles of denaturation (95°C, 15 seconds) and annealing to extension (60°C, 1 minute), following an initial Taq polymerase activation step (95°C, 10 minutes). The RNase P control reagent (Applied Biosystems) was used as an internal control for calculating the input cell number (using VIC reporter dye). DNAs extracted from TL-Om1 and normal human PBMCs were used as positive and negative controls, respectively. The HTLV-I proviral load (%) was calculated as the copy number of the pX region per input cell number. To correct the deviation of data acquired in each experiment, data from TL-Om1 (positive control) were adjusted to 100%, and the sample data were corrected accordingly by a proportional calculation.

Inverse long PCR

For clonality analysis, inverse long PCR was performed [17]. First, 1 μg of genomic DNA extracted from the FACS-sorted cells was digested with *Eco*RI and *Pst*I at 37°C overnight. Purification of DNA fragments was performed using a QIAEX2 gel extraction kit (Qiagen). The purified DNA was self-ligated with T4 DNA ligase (Takara Bio, Otsu, Japan) at 16°C overnight. The circular DNA obtained from the *Eco*RI digestion fragment was then digested

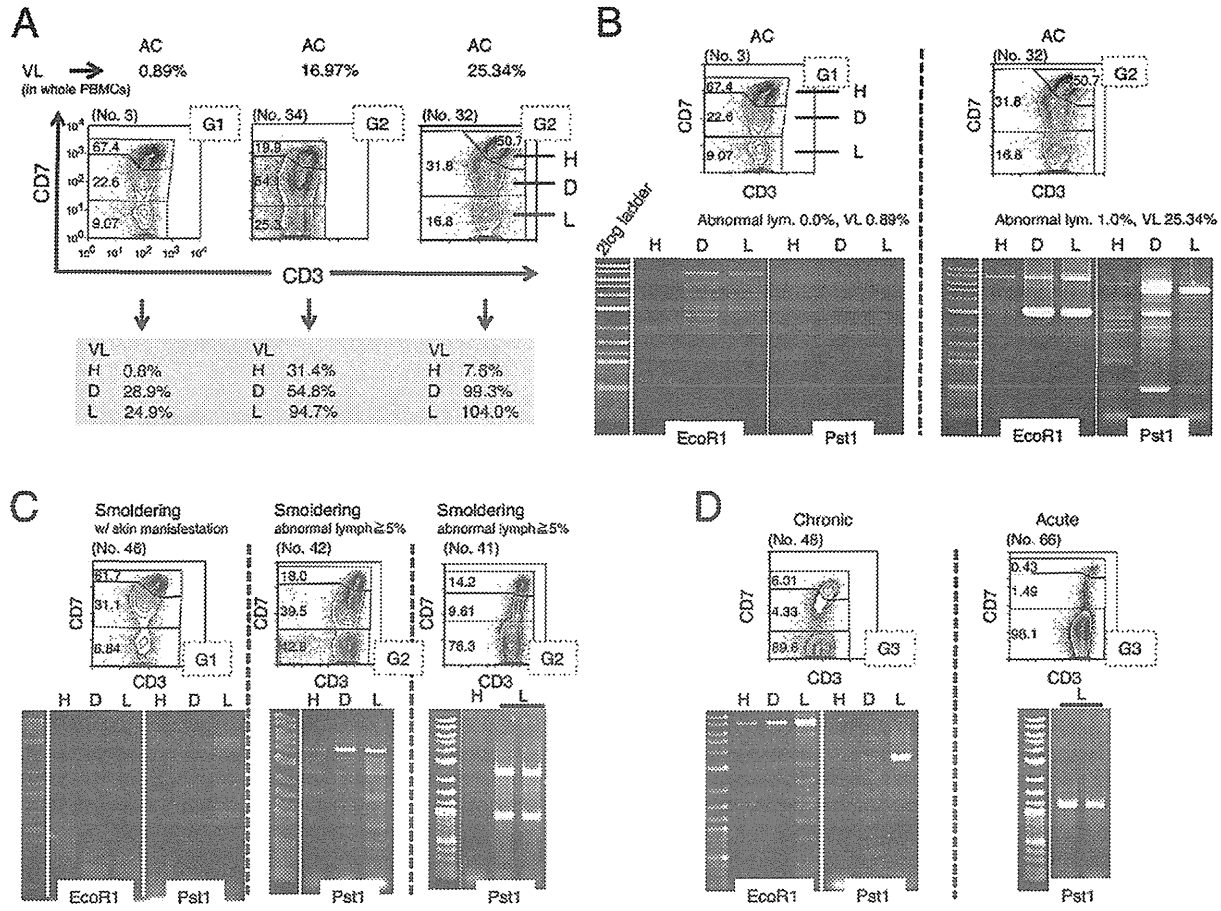

Figure 2. HTLV-I proviral load (VL) and clonality in each subpopulation, based on the CD3 versus CD7 plot. (A) The three subpopulations (H, D, L) based on the CD3 versus CD7 plot were subjected to fluorescence-activated cell sorting (FACS) and VL analysis. Three representative cases are shown. G1 or G2 in the dotted box indicates Group 1 or Group 2, categorized by the percentage of the D and L subpopulations, respectively. (B)–(D) Analysis of clonality in the three subpopulations based on the CD3 versus CD7 plot. Genomic DNA was extracted from FACS-sorted cells of each subpopulation and subjected to inverse long polymerase chain reaction (PCR). Representative data of two cases of AC (B), three cases of smoldering type, including one with skin manifestations (C), and cases of a chronic type and an acute type (D) are shown. PCR was performed in duplicate (black bars) in cases when a sufficient amount of DNA was obtained.
doi:10.1371/journal.pone.0053728.g002

with *Mlu*I, which cuts the pX region of the HTLV-I genome and prevents amplification of the viral genome. Inverse long PCR was performed using Takara LA *Taq* polymerase (Takara Bio). For the *Eco*RI-treated template, the forward primer was 5′-TGCCTGACCCTGCTTGCTCAACTCTACGTCTTTG-3′ and the reverse primer was 5′-AGTCTGGGCCCT-GACCTTTTCAGACTTCTGTTTC-3′. For the *Pst*I-treated group, the forward primer was 5′-CAGCCCATTCTATAG-CACTCTCCAGGAGAG-3′ and the reverse primer was 5′-CAGTCTCCAAACACGTAGACTGGGTATCCG-3. Each 50-μL reaction mixture contained 0.4 mM of each dNTP, 25 mM $MgCl_2$, 10× LA PCR buffer II containing 20 mM Tris-HCl and 100 mM KCl, 0.5 mM of each primer, 2.5 U LA *Taq* polymerase, and 50 ng of the processed genomic DNA. The reaction mixture was subjected to 35 cycles of denaturation (94°C, 30 seconds) and annealing to extension (68°C, 8 minutes). Following PCR, the products were subjected to electrophoresis on 0.8% agarose gels. In samples from which a sufficient amount of DNA was extracted, PCRs were performed in duplicate.

Results

CD3 versus CD7 profile in flow cytometry in various clinical subtypes of patients infected with HTLV-I

The clinical profiles of the 77 cases analyzed in this study are shown in Table 1. According to the gating procedure, as shown in Figure S1 [17], we constructed a CD3 versus CD7 plot of $CD4^+$ cells in PBMCs of various clinical subtypes from patients infected with HTLV-I and normal controls. The three subpopulations ($CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$, and $CD3^{dim}CD7^{low}$) observed are referred to as the H, D, and L subpopulations, respectively. Representative results for each clinical subtype of HTLV-I infection are shown in Figure 1A. Regarding the data for an acute-type patient (no. 66), the dominant population was the L subpopulation, in which we previously demonstrated that monoclonal ATL cells are enriched [17]. Regarding the AC (no. 19), the CD3 versus CD7 profile was close to that of the normal control, although in some AC cases, such as no. 32, the profile differed from that of the normal control, because in contrast to case no. 19,

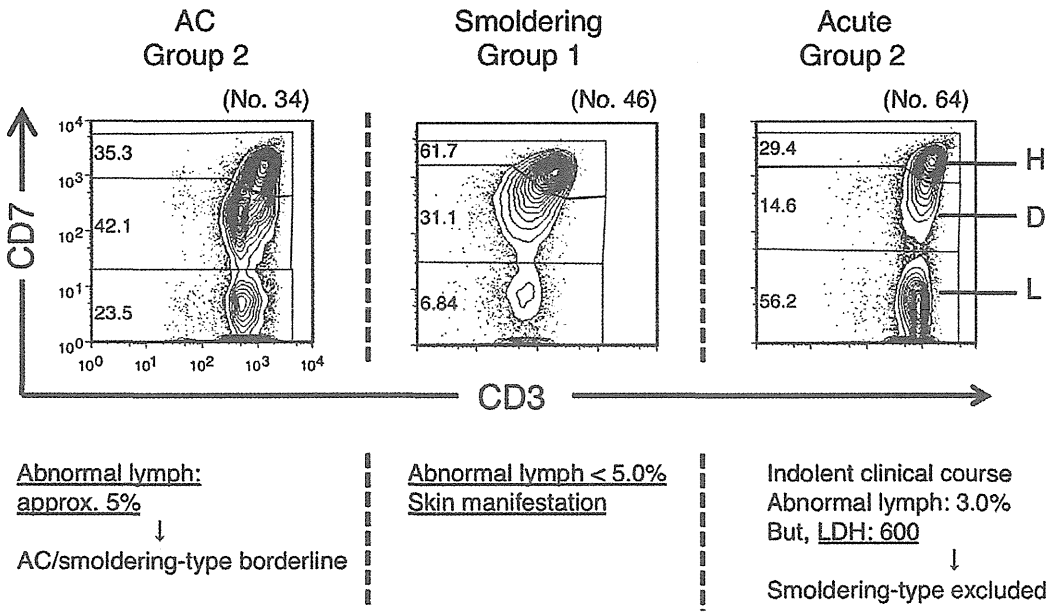

Figure 3. Study of exceptional cases categorized by proportion of the $CD3^{dim}CD7^{dim}$ (D) and $CD3^{dim}CD7^{low}$ (L) subpopulations. Left: An HTLV-I AC patient who was categorized in Group 2 in the D(%) versus L(%) plot. Middle: A patient with smoldering-type ATL who was categorized in Group 1. Right: A patient with acute-type ATL who was categorized in Group 2.
doi:10.1371/journal.pone.0053728.g003

these cases had increased D and L subpopulations. Regarding the data for indolent-type disease (smoldering and chronic), increases in the D and L subpopulations were intermediate between ACs and patients with acute-type disease. These representative flow cytometric data suggest that continuity in the CD3 versus CD7 profile seemed to exist among the various clinical subtypes of patients infected with HTLV-I.

The proportions of D and L subpopulations in all AC cases analyzed are shown in Figure 1B. Because the high HTLV-I proviral load (VL) in whole PBMCs, a VL of >4%, was reported to be a major risk indicator for progression to ATL [13], a border line was set based on VL. Group 1, the area under the diagonal line (D+L = 45%), included all AC cases with VLs of <4%. ACs with VLs of >4% were distributed between Groups 1 and 2. The proportions of D and L subpopulations in normal controls are shown in Figure S2. In this plot, all data for normal controls were distributed in Group 1. Data for all clinical subtypes are shown in Figure 1C. Most data for acute-type patients were located in the area beyond 80% of the L subpopulation and we designated this area as Group 3. Group 2, which is located between Group 1 and Group 3, included the majority of indolent-type (smoldering and chronic) cases. From these results, the three groups in the D(%) versus L(%) plot seemed to represent disease stage in each case.

Proviral load and clonality in each subpopulation in the CD3 versus CD7 plot

To further characterize each subpopulation (H, D, and L) in the CD3 versus CD7 plot, cells in each subpopulation were FACS-sorted and subjected to analysis of VL to determine the percentage of HTLV-I-infected cells in each subpopulation. Results for representative cases are shown in Figure 2A. The VL in whole PBMCs of an AC (no. 3) was low (0.89%). As expected, the VL in H, the major subpopulation, was low (0.8%). However, VLs in the D and L subpopulations were considerably higher (28.9% and 24.9%, respectively), indicating that HTLV-I-infected cells are relatively concentrated in these subpopulations. In the cases with high VLs in whole PBMCs (no. 32 with 25.34%; no. 34 with 16.97%), the VLs were also higher in the D and L subpopulations, and almost all cells in the L subpopulation were HTLV-I-infected.

In HTLV-I infection, progression to ATL requires several pathological steps, including clonal expansion [15]. To further characterize the three subpopulations based on the CD3 versus CD7 plot, we analyzed clonality in each subpopulation in patients with various clinical subtypes using the inverse long PCR method. Figure 2B shows two cases of AC. In the left case (no. 3), included in Group 1 in the D(%) and L(%) plot, multiple bands suggestive of multiple small clones were detected in the three subpopulations. However, no major band suggestive of a dominant clone was observed. In the right case (no. 32), included in Group 2, inverse long PCR of the FACS-sorted subpopulations suggested that the D and L subpopulations contained a major clone (Figure 2B). The D subpopulation had bands of the same size as those of the L subpopulation, indicating that the two distinct subpopulations contained a common major clone. Eleven cases of AC were included in Group 2. All three cases analyzed by Southern blotting (whole blood samples) were positive for clonal bands (Figure S3). In Figure 2C, data for three smoldering cases are shown. In case no. 46 (left), whose only manifestation was a skin eruption with few abnormal lymphocytes (less than 5% of white blood cells) in the peripheral blood, only faint minor bands suggestive of small clones were observed. In contrast, in the other two cases (nos. 42 and 41), intense bands suggestive of major clones were observed in both the D and L subpopulations. In no. 41 (right), weak bands were not visible, which suggested the selection of dominant clones. In Figure 2D, data for a chronic-type case and an acute-type case are shown. In both cases, intense bands in the L subpopulation suggest the existence of a major clone. The series of clonality analyses indicated that a major clone became more evident and the clinical

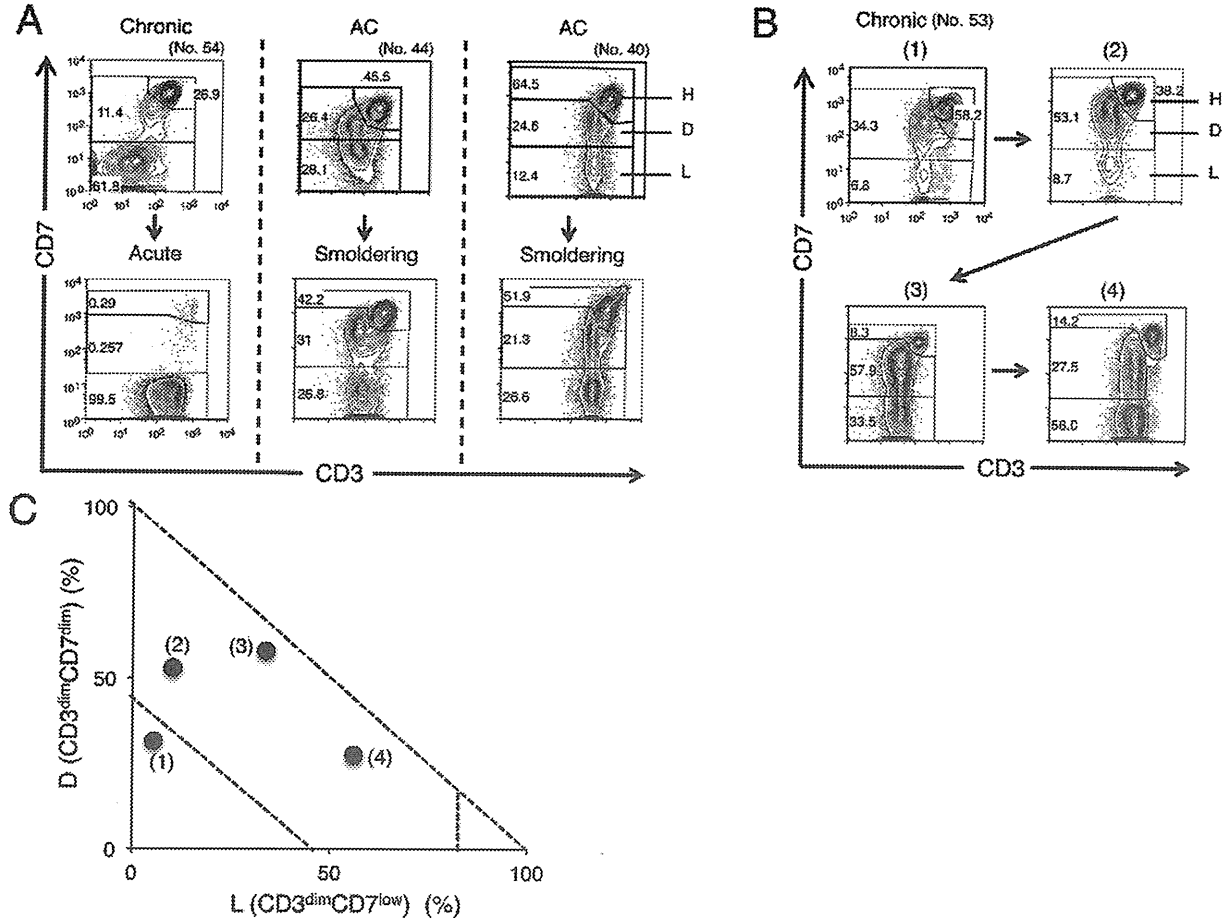

Figure 4. Alteration in the CD3 versus CD7 profile by flow cytometry in accordance with disease progression. (A) Change in the CD3 versus CD7 profile in representative cases. In all three cases shown, change in clinical data (*e.g.*, abnormal lymphocyte, LDH) resulted in progression of the clinical subtype. (B) Change in the CD3 versus CD7 profile in a time course in the case of chronic-type ATL. Clinical data are shown in Table S1. (C) Flow cytometric data in (B) are summarized in the D(%) versus L(%) plot.
doi:10.1371/journal.pone.0053728.g004

stage became more advanced as the D and L subpopulations increased.

Clinical evaluation of exceptional cases categorized by proportions of the $CD3^{dim}CD7^{dim}$ (D) and $CD3^{dim}CD7^{low}$ (L) subpopulations

As noted above, the D(%) versus L(%) plot generally represented disease stage in HTLV-I infection. However, we observed one case of chronic-type disease and three cases of smoldering-type disease in Group 1 and three cases of acute-type disease in Group 2. Furthermore, some ACs with VLs of >4% were observed in Group 2. Representative data from these apparently exceptional cases are shown in Figure 3. On the left, a case of AC (no. 34) observed in Group 2 is shown. 4.7% of lymphocytes in this blood sample were abnormal and clonality analysis by Southern blotting showed oligoclonal bands suggestive of clones of substantial size (Figure S3). These clinical data suggest that the disease stage would be around the AC/smoldering borderline. In the middle, a case of a smoldering type (no. 46) observed in Group 1 is shown. In this case, the percentage of abnormal lymphocytes in the peripheral blood was only 1%, but she had a histologically proven ATL lesion in the skin and was diagnosed with smoldering-type ATL. The other two smoldering cases categorized in Group 1 were the same as this case. These results indicate that ATL cells in these three smoldering cases infiltrated the skin, but not the peripheral blood. On the right, a case of acute-type disease categorized as Group 2 (no. 64) is shown. The clinical course of this patient was relatively indolent compared with typical acute-type disease. He had skin infiltration of ATL cells, but no lymph node swelling. However, LDH exceeded 1.5 times the upper limit of the normal range, which excludes a diagnosis of smoldering-type disease. Other acute-type cases categorized in Group 2 were diagnosed as such according to Shimoyama's criteria, but also had the same indolent clinical course as case no. 64. These cases should have been regarded as indolent ATL.

Changes in the CD3 versus CD7 profile in flow cytometry with disease progression

In several cases, we could obtain time-sequential samples (Figure 4). The patient (no. 54) shown on the left in Figure 4A progressed from chronic-type to acute-type disease. In flow cytometric analysis, decreases in the H and D subpopulations

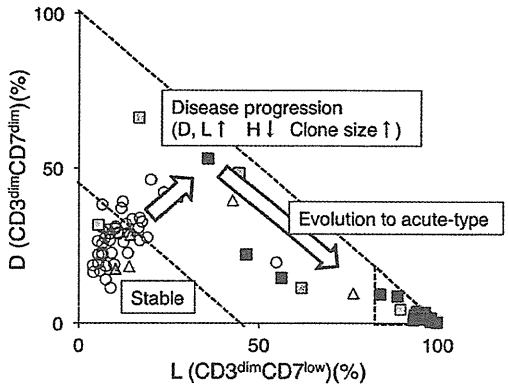

Figure 5. Summary of the study: the CD3 versus CD7 profile reflects progression of disease stage in patients infected with HTLV-I. In the percentage of D ($CD3^{dim}CD7^{dim}$) versus L ($CD3^{dim}CD7^{low}$) plot, Group 1 includes the majority of AC cases. As disease stage progresses, the CD3 versus CD7 profile then changes. With downregulation of CD3 and CD7, the D and L subpopulations increase gradually (Group 2). During this step, clones in the D and L subpopulations increase in size. Further accumulation of genetic alterations will result in rapid expansion of ATL clones—*i.e.*, evolution to acute-type ATL. In this step, the CD3 versus CD7 profile will progress from Group 2 to 3.
doi:10.1371/journal.pone.0053728.g005

and an increase in the L subpopulation were observed, indicating that disease progression correlated well with the change in the CD3 versus CD7 profile. The patients in the middle (no. 44) and on the right (no. 40) were included in Group 2 at the AC stage and later progressed to smoldering-type ATL. Although variation in the change of the flow cytometric profile was seen between these patients, the results suggest that ACs in Group 2 are at high risk of developing ATL.

The patient in Figure 4B (no. 53) was initially diagnosed with AC and later progressed to chronic-type ATL. Although the initial clinical course was stable, an increase in abnormal lymphocyte numbers was later observed, and low-dose VP-16 therapy (50 mg/day) was initiated because of hypoxemia due to lung infiltration of ATL cells. Table S1 and Figure 4C show summaries of the clinical data and the flow cytometric analyses, respectively. The flow cytometric data correlated well with disease progression.

Discussion

Findings in our previous analysis of acute-type ATL samples prompted our analysis of various clinical subtypes of patients infected with HTLV-I to examine whether the CD3 versus CD7 profile reflects the progression of oncogenesis in HTLV-I-infected cells [17]. Representative flow cytometric data shown in Figure 1A suggested that the CD3 versus CD7 profile changed during disease progression. As the disease stage progressed, the D and L subpopulations increased with concomitant decreases in the $CD3^{high}CD7^{high}$(H) subpopulation. Figure 1C, a summary of the flow cytometric data of all cases analyzed, reveals that the two-dimensional plot of the proportions of the D versus L subpopulations could divide all cases into three groups. Group 1, the area under the diagonal line, equivalent to 55% of the H subpopulation in which all normal controls were included (Figure S2), contained the majority of HTLV-I ACs. Group 3 was the area beyond 80% of the L subpopulation, and the majority of acute-type cases were included in this group. Group 2, located between Groups 1 and 3 (*i.e.*, less than 55% of the H subpopulation and 80% of the L subpopulation), included indolent-type (smoldering and chronic) cases and some AC cases. These results suggest that the CD3 versus CD7 expression profile reflects disease stage. Initially, both the D and L subpopulations gradually and simultaneously increased. However, at the clinically advanced stage, the increase in the L subpopulation was prominent. The change is considered to reflect the biological difference between the D and L subpopulations, which needs to be clarified.

In HTLV-I infection, the small clones of infected cells are considered to coexist from the AC stage [19,20]. A selected clone from the multiple small clones then grows and progresses to the malignant state, and the emergence of a dominant clone indicates disease progression in ATL [19,20]. As shown in Figure 2B–D, major bands suggesting dominant clones were evident in patients with progressed clinical subtypes or those in the advanced group in the CD3 versus CD7 profile, and major bands existed exclusively in the D and L subpopulations. These data also support the idea that increases in the D and L subpopulations correlate with the progression of disease stage. AC cases in Group 2 had high HTLV-I proviral loads (>4%; Figure 1B) and clear major bands were observed by inverse long PCR in these cases (Figure 2B, right). Sasaki *et al.* reported that two cases of HTVL-I AC with oligoclonal bands on Southern blots and high VLs (20%) had progressed to ATL by 4 and 3.5 years later [21]. The two cases may correspond to HTLV-I AC in Group 2 proposed in our study. In fact, two cases of ACs in our series that were included in Group 2 progressed to smoldering ATL (Figure 4A). AC cases in Group 2 could be regarded as advanced carriers. Our flow cytometric analysis could apparently discriminate high-risk AC cases from stable ones. Follow-up analysis of these cases is warranted to determine whether AC cases included in Group 2 progress to ATL. Flow cytometric data for these AC cases included in Group 2 (Figure 1A and 1C) were similar to those for indolent ATL cases in Group 2. These ACs in Group 2 can be considered essentially the same as smoldering ATL cases. Some of the ACs categorized according to Shimoyama's criteria should in fact be separated and regarded as a subtype together with at least some of the smoldering ATL cases.

Iwanaga *et al.* reported that high HTLV-I proviral load (>4%) in whole PBMCs was a risk factor for progression to ATL [13]. In Figure 1B, the ACs with VLs>4% were distributed between Groups 1 and 2. These findings suggest that not all ACs with high VLs are currently in an advanced stage, although they may have the potential to develop ATL in the future.

In general, the categorization by flow cytometric profile correlated well with the current classification of clinical subtypes, with some exceptional cases of acute-type and smoldering-type disease (Figure 3). The only manifestation of three smoldering cases categorized in Group 1 was skin lesions; they fell into Group 1 because they showed minimal abnormalities in peripheral blood [22]. Three acute-type ATL cases categorized in Group 2 had indolent clinical courses. A diagnosis of acute-type disease is made when the indolent-type and lymphoma-type are excluded, according to Shimoyama's criteria. The CD3 versus CD7 plot may discriminate the cases that will follow an indolent clinical course from the aggressive acute-type ATL.

The VL in each subpopulation indicated that HTLV-I-infected cells were relatively concentrated in the D and L subpopulations (representative data are shown in Figure 2A). These data are consistent with downregulation of CD3 and CD7 being relevant to HTLV-I infection, although cells without HTLV-I infection may also contribute to this change to some extent, as a substantial subpopulation of T cells has been reported to be CD7-deficient under physiological [23,24] and certain pathological conditions,