HTLV-1キャリアの現状（愛知県）

人口　7,426,411人 (H25年1月)

出生数　69,872 人/年 (H22年)

キャリアマザーの出産は 140-170人/年 程度??

年代別のキャリア陽性率と陽性者数(平成18-19年)

	0～4歳	5～9	10～14	15～19	20～24	25～29	30～34	35～39	40～44
陽性率	0.009385	0.009385	0.009385	0.093853	0.176035	0.176035	0.228327	0.228327	0.472627
陽性者数	43	46	45	470	1069	1139	2177	2196	3879

45～49	50～54	55～59	60～64	65～69	70～74	75～79	80歳以上	合計
0.472627	0.991561	0.991561	0.5	0.5	0.5	0.5	0.5	
3317	8003	11538	12381	11693	9480	6699	7896	82071

当時献血年齢は16歳～64歳までで、15～19歳は、16～19歳のデータをそのままあてはめた。
1～15歳は、16～39歳のカーブを指数関数的に延長、65～99歳は40歳以降のカーブ（ほぼ直線）をそのまま直線的に延長して推定したもの。

HTLV-1母子感染についてのアンケート

（回答は、郵送　または　FAX(052-264-0331）にて10月1日までに）

（愛知県産婦人科医会ニュース　本年8月号7頁参照）　156/294 回収率53%

1)　貴院にて妊婦に対してHTLV-1抗体を検査し始めたのは、いつ頃からですか？

i）　10年以上前から 88
ii）　5年以上前から 23
iii）　5年以内　14
iv）　公費負担になった平成23年度から 12
v）　やってない　19　(お産を扱っていない施設も)

2)　今までにHTLV-1抗体陽性妊婦がいましたか？

i)　いない　70
ii)　スクリーニング法で陽性、確認試験(Western Blot法）で陰性(104名)
iii)　スクリーニング法・確認試験で両方陽性　(105名)
iv)　確認試験で保留、PCR法で再精査　(　8　名)
v)　確認試験せず 15名

以下は、HTLV-1抗体陽性妊婦を経験した施設のみお答え下さい。
複数の症例がある場合は、３）以下を複数枚コピーしてご回答下さい。

3)　その患者さんを精査のために、妊娠中に他の病院へ紹介されましたか？

i)　自院で精査し、自院で分娩　57
ii)　里帰り分娩　3　(県名　長崎2、熊本1）
iii)　県内の専門病院へ紹介　4

4)　その症例の栄養方法は？

i)　他院のため不明 4
ii)　完全人工栄養 69、凍結・解凍した母乳27、短期母乳栄養21、母乳栄養　7
iii)　上記の栄養方法は、どのような方法で決定されましたか？
（医療機関からのアドバイス 25、　本人の希望 38、家族の希望 3）

5)　乳幼児のHTLV-1抗体の定期的なフォローアップは？

自院17　（1-3　年間フォローアップ中、　途中で脱落して不明　15）
他病院へ依頼　13
していない 5

6)　母親のATL（成人T細胞白血病）やHAM（HTLV-1関連脊髄症）を経験されたことはありましたでしょうか？　また、これらの定期的なフォローアップは？

ATL　（経験有り 3）
HAM　（経験有り　0）

他病院へ依頼3

7)　HTLV-1抗体陽性妊婦を受け持たれて、具体的に困った事項があれば、裏面に具体的にお書き下さい。

以下原文のまま

・今さら調査目的は何の為でしょうか？　検査の公費になったのも最近であり、あまりにも時期を逸していると思います。
・乳幼児のフォローアップを拒否された。
・院内関係職員、他の患者への感染予防に気をつけた。

以下同一施設
・おっぱいが3ヶ月でやめられなく、4ヶ月に入ってしまった。冷凍母乳でつなげられなかった。
・妊婦さんと話し合う課程で、家人をふくめ、けんかになって、結局妊婦さんに迷惑をかけた。（H10年頃）
・赤ちゃんが小さく、34週で母体搬送をお願いした。その折り、HTLV-1が測ってなく、母乳拒否され、ママにかわいそうな思いをさせた。（H8頃）

HTLV-1母子感染対策を推進するための調査結果

1 回答数

県保健所	12
市町村	50
政令中核市	4
計	66

2　平成23年度1年間及び平成24年4月から9月までにHTLV-1抗体陽性者からの相談状況

（１）相談箇所別、相談種別の状況（Ｎ＝６６）

	相談「あり」の施設		相談「あり」の相談種別							
			家庭訪問		電話		面接		計	
	数	率	実	延	実	延	実	延	実	延
県保健所（１２）	4	33.3	3	4	2	2	2	2	7	8
市町村（５０）	3	6.0	1	1	0	0	2	3	3	4
政令中核市（４）	2	50.0	2	2	5	5	9	9	16	16
計（６６）	9	13.6	6	7	7	7	13	14	26	28

（２）地区別、相談種別の状況（Ｎ＝６６）

	相談「あり」の施設		相談「あり」の相談種別							
			家庭訪問		電話		面接		計	
	数	率	実	延	実	延	実	延	実	延
尾張（４４）	7	15.9	3	3	5	5	9	10	17	18
三河（２２）	2	9.1	3	4	2	2	4	4	9	10
計	9	13.6	6	7	7	7	13	14	26	28

（３）相談内容（複数回答）Ｎ＝９

相談内容	施設数	相談内容	施設数

①生まれてくる子どもへの感染	3	⑧検査方法について	2				
②上の子どもへの感染	1	⑨専門医療機関について	2				
③子ども以外の家族への感染	1	⑩家族会について	0				
④生まれてくる子どもの栄養方法	2	⑪感染予防について	2				
⑤自身の発病について	3	⑫精神的な問題	1				
⑥疾患（ATL、HAM）について	1	⑬生活指導	1				
⑦家族関係について	1	⑭退院後の支援先について	1				
⑮その他							

○保健所で相談できる内容はどのようなことかの問い合わせ。

○夫から妻へ感染し、その後妊娠したため、母（妻）自身がショックを受けておられた。主に母（妻）の精神的フォローを中心に関わっている。

○経過観察受診のタイミング

○ＨＡＭは、特定疾患医療給付の対象となるか。

○養育医療申請や未熟児訪問時の面接にて、母がHTLV-1陽性者であることを聞き、感染や栄養方法の把握をした。それらについて、母は受けとめており、心配や不安がないことを確認した。

（４）相談を受ける上で困った内容（複数回答）Ｎ＝９

内　容	施設数	内　容	施設数
①生まれてくる子どもへの感染に対する相談	1	⑧検査方法についての相談	0
②上の子どもへの感染に対する相談	1	⑨専門医療機関についての相談	1
③子ども以外の家族への感染に対する相談	1	⑩家族会について相談	0
④生まれてくる子どもの栄養方法についての相談	1	⑪感染予防についての相談	1

	⑤自身の発病についての相談						1	⑫精神的な問題					1	
	⑥疾患（ATL、HAM）についての相談						1	⑬生活指導についての相談					0	
	⑦家族関係についての相談						0	⑭退院後の支援先についての相談					0	
								困ったことなし					2	
	⑮その他													
	○夫から感染したことで、夫への不信感が募ってしまった。													
	○産院からは、子どもの栄養方法に対する指導・助言のみで、母自身の健康管理に対する指導はなかったとのことであった。（「普通の生活でよい」とのみ。）経過観察のため、血液内科の受診を勧めたが、専門医が分からず具体的な病院の選択は母に委ねる結果となった。また、母に不安を与えすぎないように言葉を選ぶのに困った。													
	（５）医療機関からの連絡の有無（Ｎ＝９）													
		施設数												
	あり	2												
	なし	7												
	３　相談支援体制について													
	（１）感染が分かった妊婦への必要な支援内容（複数回答）Ｎ＝６６													
	内　容							施設数						
	①妊婦自身が納得して栄養方法を選択するための支援							59	89.4					
	②選択した栄養方法が確実に実施できるような支援							58	87.9					
	③自身の発病に関する相談支援							57	86.4					
	④子どもの感染に関する相談支援							59	89.4					
	⑤家族への指導・相談支援							55	83.3					
	⑥専門相談医療機関の整備							60	90.9					
	特になし							3	4.5					

	⑧その他
	○保健機関での支援については、育児支援が中心であると思われる。
	○妊婦や家族が、栄養方法や母自身の健康管理、また、家族との関係性等総合的専門的な相談が継続的に受けられる支援が必要と思います。
	○行政と医療機関と役割分担が必要であると思います。
	○⑥は市町村の仕事ではないと思います。
	○この管内では、陽性者が少ない現状があります。陽性者がいた病院とは日頃から連絡会議があったり連携が取れていますが、連絡がありませんでした。【最終結果陰性だったのかも】 外来看護師さんも主治医にお任せ的な対応なので、しっかりフォローしていくにはきちんとしたシステム化が必要かと思います。その前に、いかに支援していくか保健所と市町保健師間での地域での支援についての検討も必要かと思います。調査結果も参考に周産期関係機関連携会議での議題としてもいいかと思います。
	○医師から、必要以上に不安を与えないように病気の正しい知識を伝えることが重要だと思います。
	○支援の必要性は感じているが、HTLV-1抗体陽性妊婦・産婦の把握が不十分であり、地域における相談支援の体制は整っていない状況にある。相談従事者の知識及び技術の向上も含めて必要な支援体制を整備していけるとよい。

（２）母子感染予防及び相談支援体制としてどんなことが必要と思うか。（複数回答）Ｎ＝６６

内　容	施設数	
①相談窓口の整備	62	93.9
②専門医療機関の明確化	61	92.4
③地域の医療機関と専門医療機関との連携	58	87.9
④医療機関と保健機関の連携	60	90.9
⑤検査体制のマニュアル化	47	71.2
⑥地域における相談支援	50	75.8
⑦保健・医療機関の従事者の知識・支援技術の向上	60	90.9
特になし	1	1.5

	⑧その他
	○感染者であることの不安や子供へ感染の不安から、育児不安につながる可能性がある。医療機関との連携が重要と考える。

	○まずは、専門医療機関の明確化と総合的な専門相談窓口が必要と思います。
	○行政と医療機関と役割分担が必要であると思います。
	○HTLV-1検査について実施の有無の結果把握のみ。母から相談がある時のみの対応になる。
	○発病までの経過が、相談をふくめ見守られるような、かかりつけの地域の医療機関と専門医療機関との連携が必要だと思います。
	○医療機関からの妊婦健診の結果報告にて、HTLV-1抗体陽性の有無の記載を明確にし、早期支援に繋げられるとよい。 また専門医療機関を明確にし、医療間及び医療と保健の連携を充実させ、情報提供及び相談支援体制を整備していけるとよい。
	○検査体制のみでなく、支援体制や支援方法についても一定のマニュアルが示されると、安定した支援がなされるのではと思います。
	○フローチャート等の作成により、関係機関の役割を明確にし、支援体制が可視化されるとよい。
	○支援体制の向上のため、研修の継続
	○HTLV-１の母子感染を予防するため、妊婦健診結果で把握した場合に適切な指導・支援することが必要であり、医療機関と保健機関の連携が必要
4　その他	
	○母の感染、疾病への不安ひいては育児不安に対しての支援は地域の保健機関の役割だと思っています。専門医療機関と連携して支援を行うため、専門医療機関の情報を提供してもらいたいです。
	○HTLV-1への理解があまりないのではないかと感じられる母に対して、専門医療機関（母自身の主治医）が不在の状況下で、理解を促す説明をしたとして、その不安をしっかり受け止め続けられる自信が持ちきれていないのが現状です。
	○HTLV−１抗体検査の結果が主治医と妊婦の間にとどめられているのが現状であれば、相談支援は医療機関が主となって実施するのがよいと思います。市町村で相談時は対応しますが、専門の相談窓口については、各保健所で実施していただくのがよいと思います。
	○妊婦自身の健康に関する相談やフォロー状況については、地域では把握しにくい場合もあるので、継続して医療機関で相談やフォローをしてほしい。
	○ＨＴＬＶ−１陽性者を町内でフォローしていく際の基盤、連携が不十分である。保健従事者の知識の向上と、専門医療機関との連携が必要である。今後も研修や情報提供の場を設けていただきたい。
	○ＨＴＬＶ−１抗体検査の結果は、市町村代表と医師会との話し合いの結果、実施の有無と実施日のみの記載と決められ、それに従い実施しているもの
	○HTLV-１母子感染対策について地域・保健機関に求める役割とは何でしょうか。
	○地域・保健機関での支援体制をつくられていくのであれば、医療機関から検査結果の詳細（検査値・異常の有無）について情報共有できる体制も検討していく必要がある

	○乳児家庭全戸訪問にて、母がHTLV-1キャリアで、母乳栄養を選択肢し研究協力のために医療機関を受診しているときいた。保健師自身にHTLV-1の予防や実際の対応方法などの知識が乏しかったため、この調査により保健機関でも学ぶ機会が増えると良いと感じた。
	○当保健所では現在のところ相談等はありませんが、相談があった場合を考えると相談先の明確化、相談体制の整備が必要と思います。
	○妊婦健診で実施しても結果は町には知らされないため実態が良くわかっていないのが現状です。
	○HTLV－１の検査実施時期につきましては、国は妊娠10週以降から妊娠30週頃までの検査を薦めております。初回検査で他の感染症検査と合わせて実施する医療機関もあるようですが、治療法もない感染症であり、妊婦自身の出産・育児への精神的、身体的な準備が整った時期での検査の実施により、継続した保健指導が可能になると考えます。
	○妊婦健診を受けずに出産された方の検査体制
	○妊婦健診の結果については、HTLV-１の実施の有無のみであるため、効果的な母子感染予防につながるよう医療機関からの情報把握の方法と出産後の支援が円滑にできるような体制づくりが必要
	○対象者に指導するためのわかりやすいパンフレットが必要
	○キャリアの妊婦がどんな栄養方法を選択するのか、その方法を選択するまでの支援や出産後、選択した栄養方法が実施できるように、また母のメンタル面を支援していくためにも身近な保健部門で支援できるよう医療機関と保健機関が連携を取る必要があると思う。

５　妊婦健康診査におけるＨＴＬＶ－１抗体検査の結果の把握状況（Ｎ＝５４）

	施設数
把握している	19
把握していない	35

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
内丸 薫	The CD3 versus CD7 plot in multicolor flow cytometry reflects progression of disease stage in patients infected with HTLV-I.	PLoS One	8	doi:10.1371	2013
内丸 薫	成人T細胞白血病・リンパ腫の多彩な肺病変	血液内科	66(5)	in press	2013
山野嘉久、他	HTLV-1関連脊髄症（HAM）の治療法を確立していくために―その現状と展望―	日本臨牀	70(4)	705-713	2012
岡山昭彦	特集/ストップ　ザ　性感染症　性感染症―診断・治療　HTLV-1 感染.	臨床と研究	89(7)	907-10	2012
渡邊清高、他	がん情報の普及に向けたわが国の政策とがん拠点病院の役割	保健の科学	54(7)	436-446	2012
齋藤 滋	シンポジウム2「HTLV-I母子感染」HTLV-I検査が全国で行なわれるようになった経緯.	日本周産期・新生児医学会雑誌	48	in press	
齋藤 滋、板橋家頭夫	シンポジウム2「HTLV-I母子感染」座長のまとめ.	日本周産期・新生児医学会雑誌	48	in press	
齋藤 滋	HTLV-1母子感染対策のために助産師が知っておきたい知識	ペリネイタルケア	31	65-71	2012
石田高司、鵜池直邦、宇都宮 與、他	Allogeneic hematopoietic stem cell transplantation for adult T-cell leukemia-lymphoma with special em-phasis on preconditioning regimen:a nationwide retro-spective study.	Blood	120(8)	1734-1741	2012

Ⅳ．研究成果の刊行物・別刷

OPEN ACCESS Freely available online

PLOS ONE

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

Seiichiro Kobayashi[1], Yamin Tian[1,2], Nobuhiro Ohno[3], Koichiro Yuji[3], Tomohiro Ishigaki[4], Masamichi Isobe[3], Mayuko Tsuda[3], Naoki Oyaizu[5], Eri Watanabe[4], Nobukazu Watanabe[4], Kenzaburo Tani[2], Arinobu Tojo[1,3], Kaoru Uchimaru[3]*

1 Division of Molecular Therapy, Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **2** Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, **3** Department of Hematology/Oncology, Research Hospital, Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **4** Laboratory of Diagnostic Medicine, Division of Stem Cell Therapy, Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **5** Clinical Laboratory, Research Hospital, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

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.

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* E-mail: uchimaru@ims.u-tokyo.ac.jp

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.
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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 QJAamp 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′-

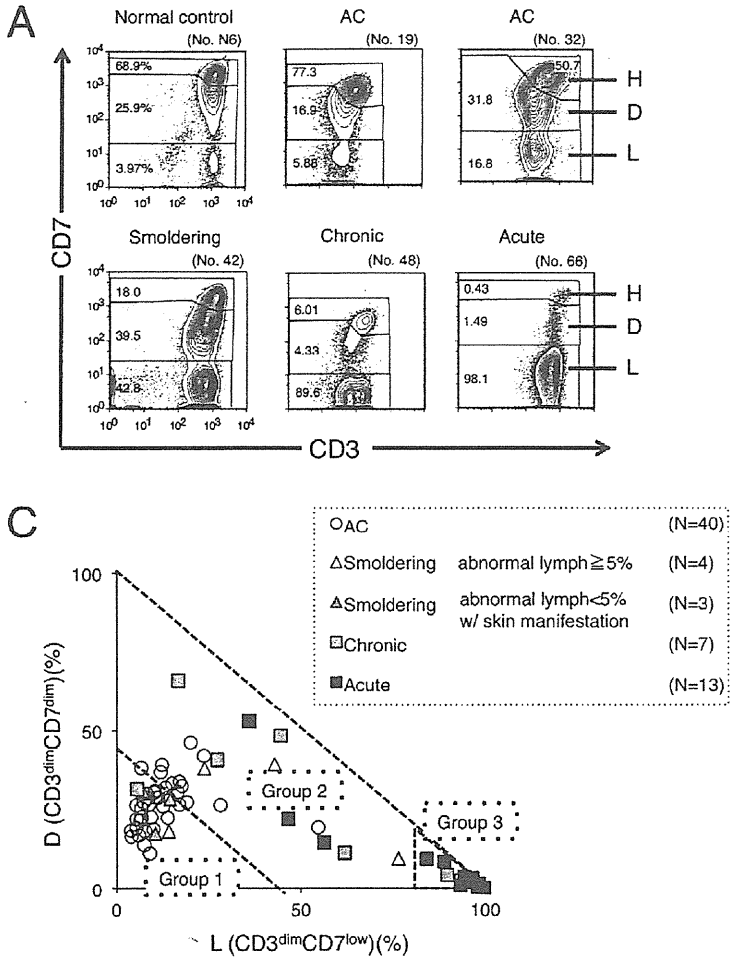

Figure 1. CD3 versus CD7 plots in flow cytometric analysis of patients who are asymptomatic HTLV-I carriers (ACs) and have various clinical subtypes of adult T-cell leukemia-lymphoma (ATL) suggest disease progression in HTLV-I infection. (A) Flow cytometric profile of an AC, various clinical subtypes 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.
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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

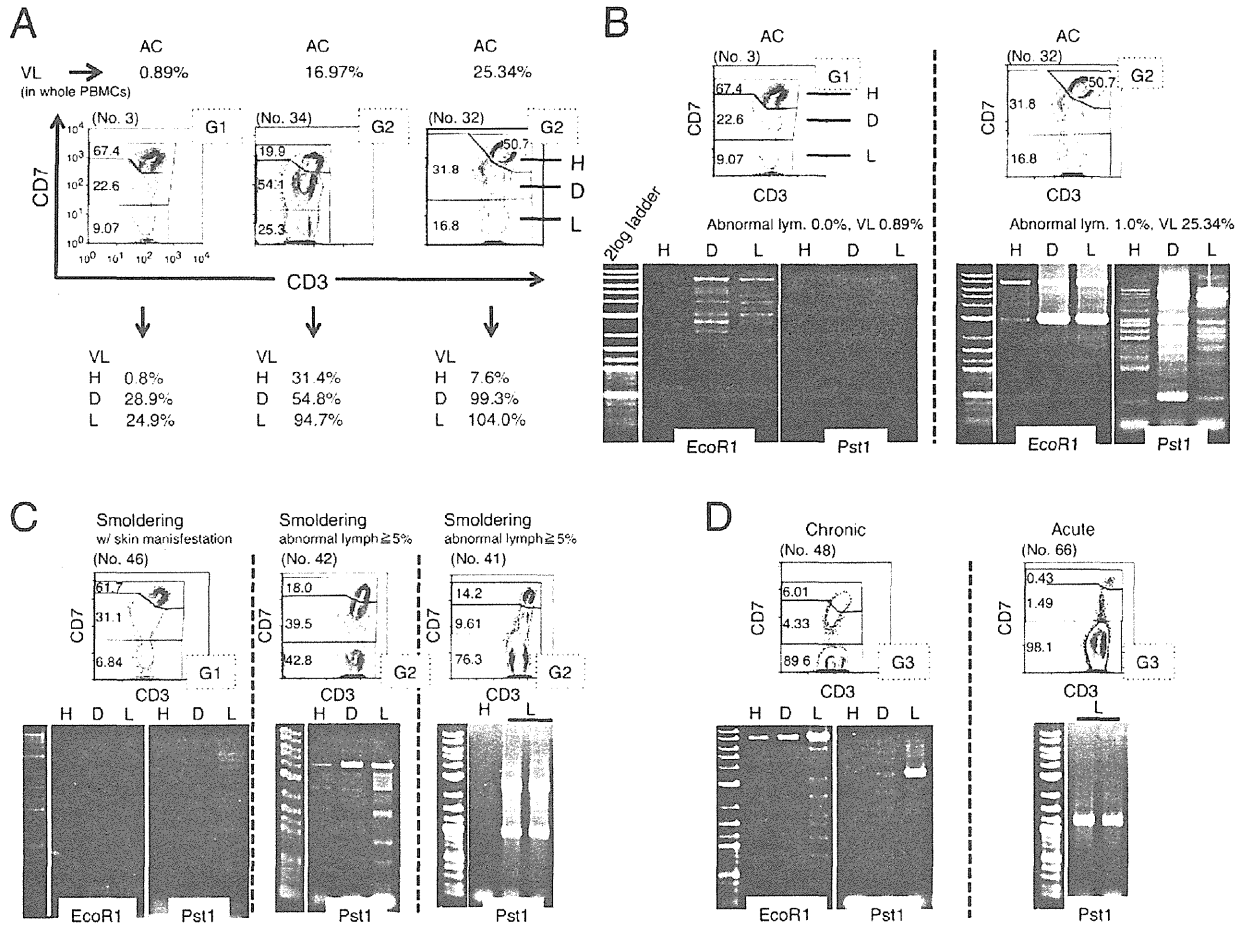

Figure 2. HTLV-I proviral load (VL) and clonality in each subpopulation, based on the CD3 versus CD7 plot. (A) The three subpopulations (H, D, L) based on the CD3 versus CD7 plot were subjected to fluorescence-activated cell sorting (FACS) and VL analysis. Three representative cases are shown. G1 or 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.
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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,

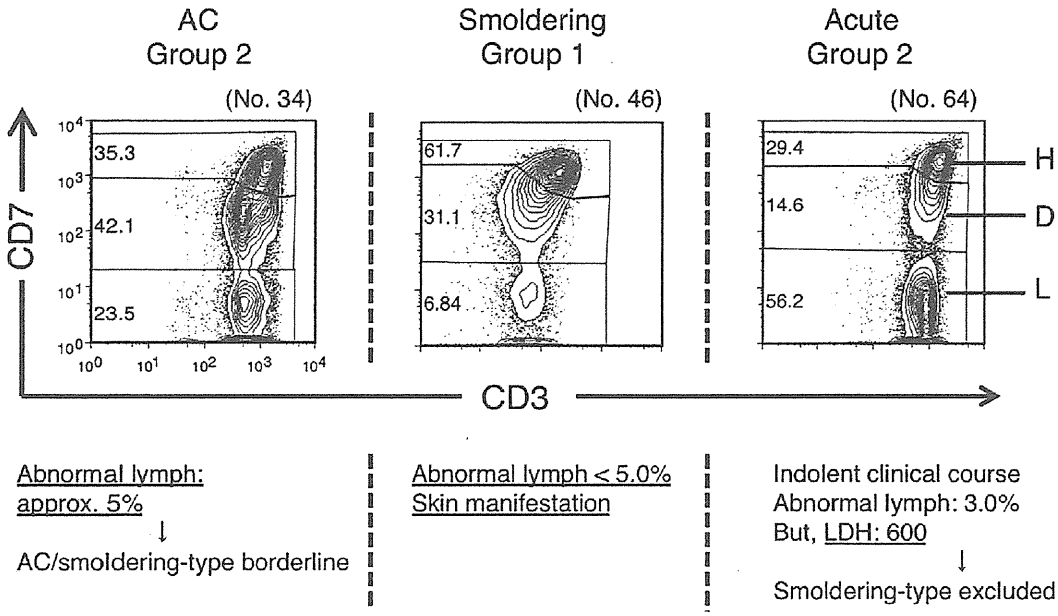

Figure 3. Study of exceptional cases categorized by proportion of the $CD3^{dim}CD7^{dim}$ (D) and $CD3^{dim}CD7^{low}$ (L) subpopulations. Left: An HTLV-I AC patient who was categorized in Group 2 in the D(%) versus L(%) plot. Middle: A patient with smoldering-type ATL who was categorized in Group 1.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

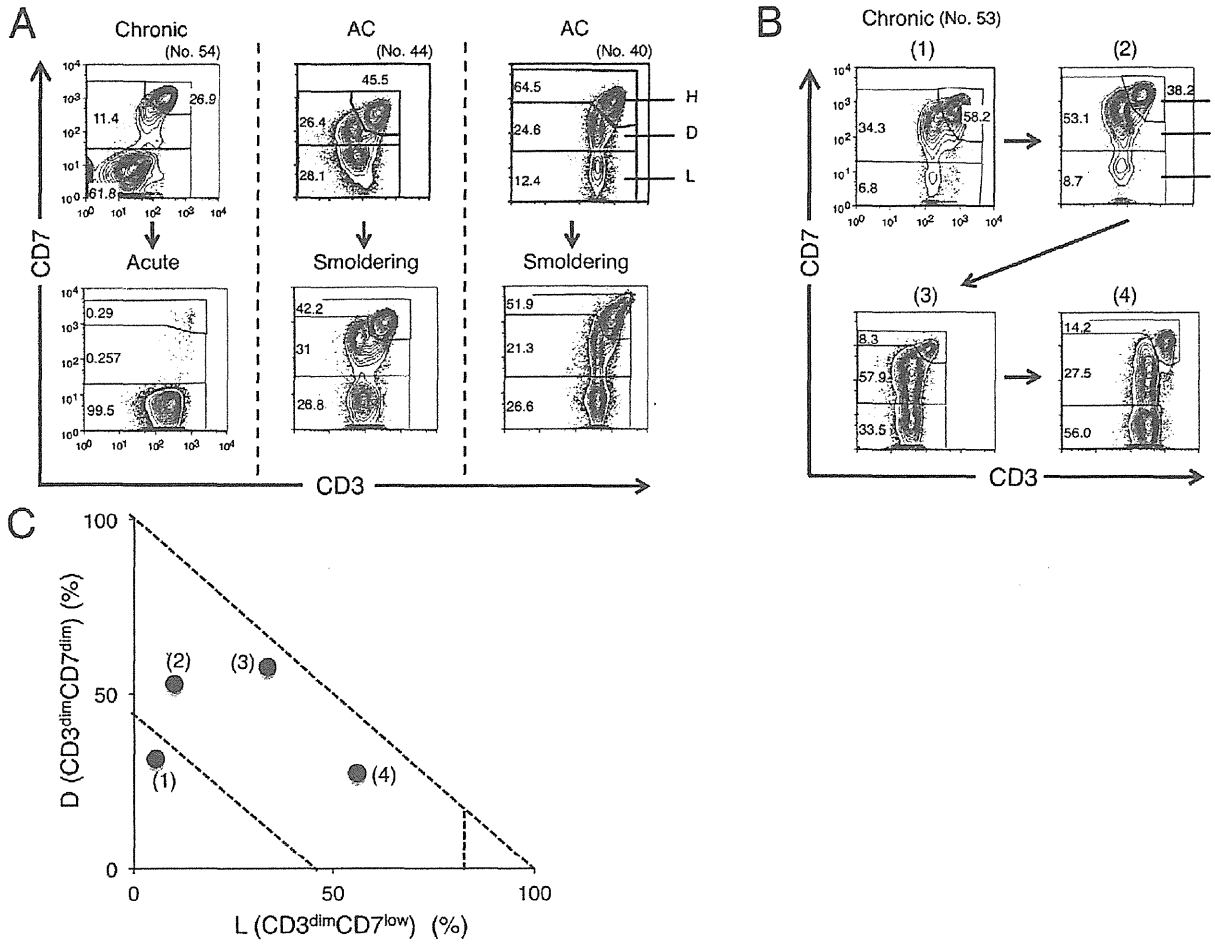

Figure 4. Alteration in the CD3 versus CD7 profile by flow cytometry in accordance with disease progression. (A) Change in the CD3 versus CD7 profile in representative cases. In all three cases shown, change in clinical data (*e.g.*, abnormal lymphocyte, LDH) resulted in progression of the clinical subtype. (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.
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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

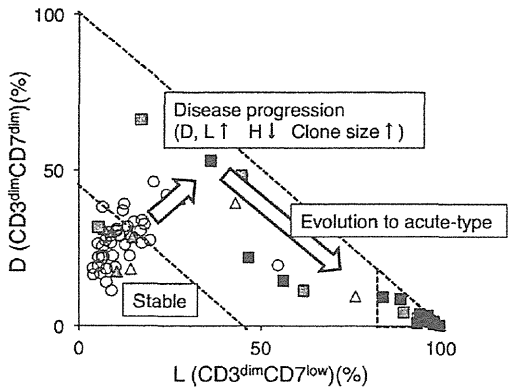

Figure 5. Summary of the study: the CD3 versus CD7 profile reflects progression of disease stage in patients infected with HTLV-I. In the percentage of D ($CD3^{dim}CD7^{dim}$) versus L ($CD3^{dim}CD7^{low}$) plot, Group 1 includes the majority of AC cases. As disease stage progresses, the CD3 versus 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,
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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,

including autoimmune disorders and viral infection [25–29]. To more precisely analyze phenotypic changes in HTLV-I-infected cells, markers that indicate HTLV-I infection should be incorporated in future studies.

A summary of this study is shown in Figure 5. In the CD3 versus CD7 profile, most AC cases were included in Group 1, in which the D and L subpopulations were relatively small. Consistent with disease progression to smoldering- and chronic-type ATL, a decrease in the H subpopulation and increases in the D and L subpopulations occur (Group 2). In this step, increases in the sizes of clones in the D and L subpopulations are observed. Further expansion of the leukemic clone results in progression to acute-type ATL in which the L subpopulation has expanded (Group 3). According to a study by Yamaguchi *et al.*, the natural course of ATL is to progress from the HTLV-I carrier state through the intermediate state, smoldering ATL, and chronic ATL, and finally to the acute ATL, indicating a process of multistage leukemogenesis [19]. We consider this study to successfully link the progressive clinical status and phenotypic changes in HTLV-I-infected cells. However, the way in which this profile reflects multistep oncogenesis in HTVL-I infection at the molecular level remains unclear. Further molecular analyses of the three subpopulations will help in understanding the mechanism(s).

Supporting Information

Figure S1 Representative flow cytometric analysis of an HTLV-I asymptomatic carrier (patient no. 32). The CD3 versus CD7 plot of $CD4^+$ cells was constructed according to the gating procedure shown in this figure. In the plot, we designated three subpopulations: H ($CD3^{high}CD7^{high}$), D ($CD3^{dim}CD7^{dim}$), and L ($CD3^{dim}CD7^{low}$).
(PPTX)

Figure S2 A two-dimensional plot of 10 normal controls showing the percentage of the D and L subpopulations.
(PPTX)

Figure S3 Southern blot analysis of clonal integration of the HTLV-I provirus. Representative data (AC, No. 34) are shown. In *Eco*RI or *Pst*I digestion, a band indicated by a red arrow represents the monoclonal integration of the provirus. The band pattern indicates that two major clones coexist. This analysis was performed by a commercial laboratory (SRL, Tokyo, Japan).
(PPTX)

Table S1 Clinical data in a case of chronic-type ATL (No. 53). Proportion of abonormal lymphocytes in the preipheral blood WBC were evaluated by morphological exmanination. LDH: Lactate dehydrogenase (normal range, 120–240 U/L) sIL-2R: soluble interleukin-2 receptor (normal range, 122–496 U/ml).
(XLSX)

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Author Contributions

Conceived and designed the experiments: KT AT KU. Performed the experiments: SK YT. Analyzed the data: EW NW TI NO. Contributed reagents/materials/analysis tools: MI MT KU NO. Wrote the paper: SK KU.

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