

オフィス ギネコロジー—女性のプライマリ・ケア

【IV章 妊婦】

母子感染の管理

—④HTLV-1—

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# 母子感染の管理

## ④ HTLV-1

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- ◆ HTLV-1 は ATL や HAM の原因ウイルスであり、そのキャリアは全国に拡散している。
- ◆ HTLV-1 キャリア妊婦から児への母子感染の主要経路は母乳感染である。
- ◆ 人工栄養は HTLV-1 の母子感染への予防効果が最も高い栄養法である。
- ◆ 医療者は、出生後の栄養法の選択が児にとって将来の ATL の発症リスクを最も効果的に回避しうるチャンスであることに留意して、保健指導を行う。
- ◆ HTLV-1 の検査システムには、結果の告知とその後のフォロー体制の確立が重要である。

### はじめに

ヒト T 細胞白血病ウイルス-1 型 (HTLV-1) は、難治性疾患である成人 T 細胞白血病リンパ腫 (ATL) や HTLV-1 関連脊髄症 (HAM) の原因ウイルスであり、その主な感染経路は母乳を介した母子感染である。HTLV-1 キャリアは九州・沖縄地方など一部の地域で高率に認められていたが、最近は大都市圏にも拡散し、全国でその数は 108 万人と推定される。そこで、全国的な母子感染対策として、2011 年 4 月から妊婦の HTLV-1 抗体スクリーニングが全国展開され、これまで HTLV-1 キャリアが低頻度であった地域でも、妊婦の HTLV-1 抗体スクリーニングシステムの確立が必要とされている。

本稿では、長崎県における HTLV-1 母子感染予防の取り組みとその成果について概説する。

### HTLV-1 キャリアと

#### その関連疾患

レトロウイルスである HTLV-1 に持続感染しているヒトを HTLV-1 キャリアという。HTLV-1 の標的は T リンパ球であり、HTLV-1 キャリアの

血液中には感染リンパ球が存在する。しかし、B 型肝炎ウイルスキャリアや HIV キャリアの血清中には大量のウイルス粒子が認められるのに対して、HTLV-1 キャリアのそれにはウイルス粒子はほとんど認められない。HTLV-1 感染は感染細胞と標的細胞との直接接触により拡大する。感染経路として、主に母乳を介した母子感染、血液を介した輸血感染、あるいは精液を介した性交感染がある。日本国内の HTLV-1 に感染している無症候性のキャリアは約 108 万人と推定される。以前は九州・沖縄など一部の地域に偏在していると考えられていたが、最近では人口移動などの影響で大都市圏にも拡散している<sup>1)</sup>。

#### ■ATL

ATL は、主に 40 歳以上の成人に発病する白血病・リンパ腫の一種であり、①全身倦怠感・発熱などの全身症状、②リンパ節腫脹、③発疹、④免疫低下に伴う各種感染症、⑤高カルシウム血症などの臨床症状を呈する。HTLV-1 が T リンパ球に感染すると、40 年以上の長い潜伏期間を経て、あるとき HTLV-1 が感染した T リンパ球が腫瘍化し ATL を発症する。いったん ATL を発症すると治療成績は悪く 5 年生存率は約 20% である。死亡原因の多くは腫瘍死もしくは感染症による。

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表 1 栄養法別にみた HTLV-1 母子感染率 (文献 3 より引用, 改変)

栄養法	総数	陽性例	陰性例	HTLV-1 抗体陽性率
人工栄養	962	23	939	2.4%
短期母乳栄養	169	14	155	8.3%
長期母乳栄養	346	71	275	20.5%

キャリアが ATL を発症する割合は年間 1,000 人に 1 人, 生涯発症率は男性 15 人に 1 人 (6.5%), 女性は 50 人に 1 人 (1.9%) である<sup>2)</sup>. ATL のほとんどは母子感染によるキャリアから発症する<sup>2,3)</sup>.

## ■HAM

HAM は HTLV-1 が感染したリンパ球が脊髄に炎症・変性を起こし, ①両下肢の痙性麻痺, ②膀胱直腸障害, ③手足のしびれや灼熱感を呈する疾患であり, 2009 年から厚生労働省難病対象疾患に指定された. キャリアが HAM を発症する割合は年間 30,000 人に 1 人である<sup>2)</sup>. HAM が直接の死因になることはほとんどなく, ステロイド療法やインターフェロン療法によって一部の例では症状の改善を認めるが, 根本的な治療方法は確立されていない<sup>1)</sup>. HAM は母子感染による HTLV-1 キャリアのみならず性交感染や輸血感染によるキャリアからも発症する<sup>2,3)</sup>.

## ●妊婦への HTLV-1 抗体 ●スクリーニングの重要性

ATL のほとんどは母子感染によるキャリアから発症する<sup>2,3)</sup>. ATL の有効な治療法はいまだ存在しないため, その対策の主眼は一次予防である母乳感染の回避に置かれる. つまり, 現時点で ATL の危険性から次世代の子どもたちを救う最も有効な対策は, 妊婦の HTLV-1 キャリアをスクリーニングして, 陽性妊婦は母乳栄養を回避することである<sup>2~4)</sup>.

長崎県の調査では, 人工栄養を選択した HTLV-1 キャリア妊婦から出生した児の母子感染率は 962 名中 23 名 (2.4%), 授乳期間が 6 か月未満の短期母乳栄養児では 169 名中 14 名 (8.3%), 6 か月以上の長期母乳栄養児では 346

名中 71 名 (20.5%) であり, 人工栄養 vs 短期母乳, 短期母乳 vs 長期母乳, 人工栄養 vs 長期母乳のすべての比較で統計学的に有意差が認められ (表 1)<sup>2,3)</sup>, 児の母乳への曝露期間が長いほど母子感染率が上昇するという成績であった<sup>2,3)</sup>.

## ●HTLV-1 キャリアの診断法

### ■検査法の種類

HTLV-1 感染の診断には, 血清中の HTLV-1 特異抗体を同定する抗体検査, あるいは血液リンパ球中の HTLV-1 プロウイルスを同定する DNA 検査を用いる.

#### ●抗体検査法

ゼラチン粒子凝集法 (PA 法), 化学発光酵素免疫測定法 (CLEIA 法), 蛍光抗体法 (IF 法), ウエスタンブロット法 (WB 法) がある. PA 法や CLEIA 法などの簡便な検査法は, スクリーニング検査として用いられる. WB 法はウイルスの HTLV-1 抗原構成蛋白に対する抗体を検出して判定するため, ほかの抗体検査法と比較して特異度が高く, 確定診断として用いられる<sup>2,3)</sup>. Env 抗原蛋白 (gp46) と gag 抗原蛋白 (p19, p24, p53) に対するバンドをそれぞれ 1 つ以上検出した場合は陽性, まったくバンドを認めない場合は陰性, それ以外のパターンは判定保留と診断される<sup>2)</sup>.

#### ●DNA 検査

リアルタイム PCR 法は DNA の検出と定量性に優れた検査法であり, WB 法で判定保留と診断された例には, 保険適用外であるが PCR 法による診断を考慮することができる<sup>2)</sup>. また, ウイルス量の多い HTLV-1 キャリアでは ATL の発症リスクが高いことから, リアルタイム PCR 法を用いた DNA 検査のニーズは高まると思われる.

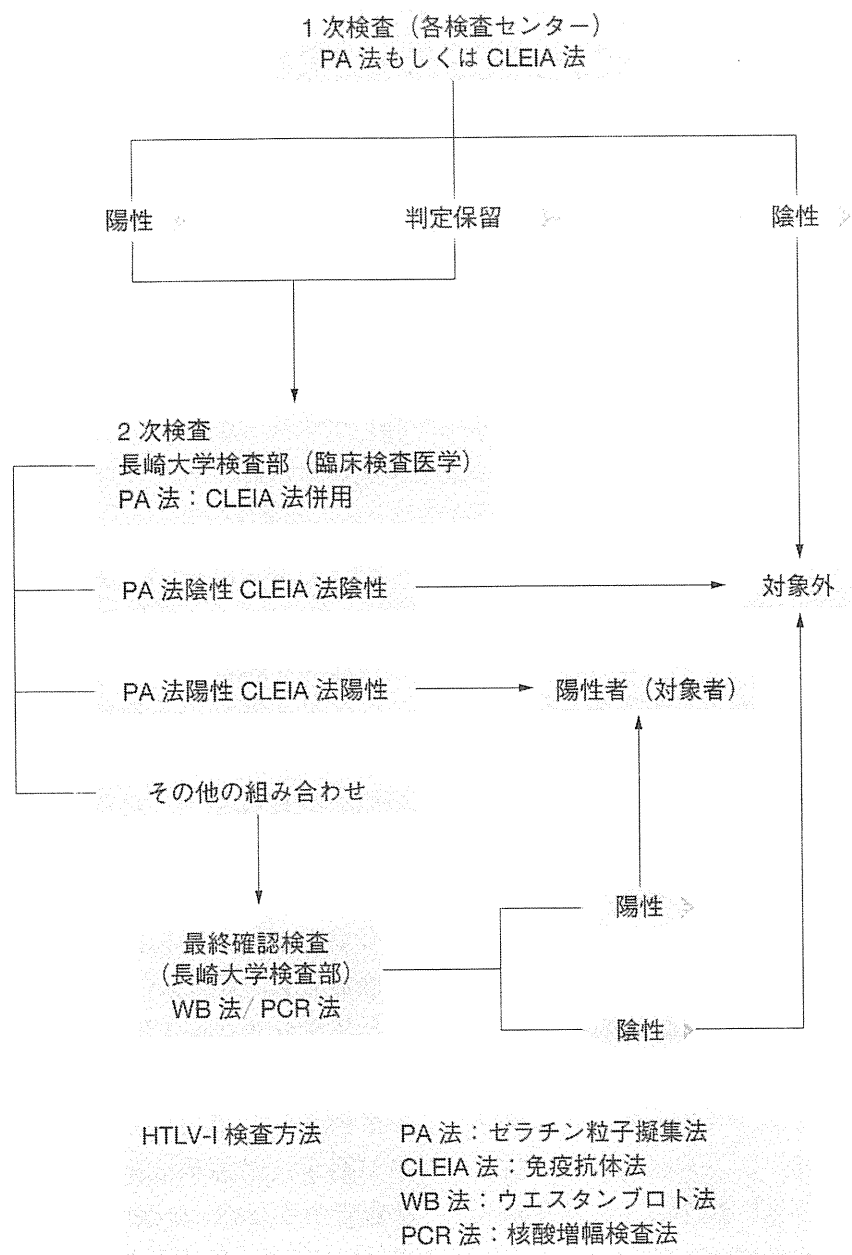


図1 妊婦のHTLV-1スクリーニング判定基準：長崎方式（文献2より）

### ■妊婦のHTLV-1抗体スクリーニングシステム（長崎方式）

長崎県では長崎県ATLウイルス母子感染防止研究協力事業連絡協議会（会長：増崎英明，長崎大産婦人科教授）を組織して，妊婦のHTLV-1スクリーニング検査システムを確立している（図1）。まず，一次検査として各診療所や病院が契約している検査センターでPA法もしくはCLEIA法を行う。そして，陽性もしくは判定保留と診断されたすべての検体は長崎大学病院検査部に集められ，確認検査としてWB法を行い，

WB法で判定保留の例にはPCR法を行って陽性もしくは陰性と最終判定している。最終判定の結果は，長崎大学病院検査部から各診療所もしくは病院に返送される。

長崎県では，1987～2010年の24年間に255,337例の妊婦をスクリーニングして9,564例が確認検査まで施行され，最終的に8,141例がHTLV-1キャリアと確定診断された（表2）<sup>2,5)</sup>。HTLV-1の一次検査には偽陽性が一定の頻度で出現するため，必ず確定診断としてのWB法を行うことが重要である<sup>2)</sup>。また，HTLV-1キャ

表 2 長崎県における妊婦の HTLV-1 抗体スクリーニング成績：1987～2010 年度（文献 3 より引用，改変）

年	出生数 (A)	妊婦抗体検査数 (B)	妊婦検査率 (B) / (A) %	精密検査数 (C)	抗体陽性者数 (D)	妊婦抗体陽性率 (D) / (B) %
1987	18,962	—	—	511	390	—
1988	18,233	9,579	52.5	1,076	691	7.2
1989	17,256	15,241	88.3	856	702	4.6
1990	16,517	14,504	87.8	804	658	4.5
1991	16,671	14,221	85.3	725	630	4.4
1992	16,036	12,123	75.6	677	566	4.7
1993	15,769	10,422	66.1	600	513	4.9
1994	15,951	9,814	61.5	523	477	4.9
1995	14,780	11,820	80.0	463	420	3.6
1996	15,182	12,966	85.4	396	342	2.6
1997	14,431	12,273	85.0	321	310	2.5
1998	14,672	12,403	84.5	356	339	2.7
1999	14,121	12,008	85.0	290	279	2.3
2000	14,098	10,286	73.0	287	276	2.7
2001	13,871	11,279	81.3	242	229	2.0
2002	13,260	10,724	80.9	230	211	2.0
2003	12,971	10,493	80.9	197	180	1.7
2004	12,947	9,990	77.2	184	177	1.8
2005	12,148	8,987	74.0	133	127	1.4
2006	12,410	8,909	71.8	144	138	1.5
2007	12,175	8,713	71.6	139	129	1.5
2008	12,173	8,930	73.4	131	124	1.4
2009	11,838	9,654	81.6	134	114	1.2
2010	13,340	9,998	74.9	145	119	1.2
合計	349,812	255,337	73.0	9,564	8,141	2.9

リアが低頻度の地域では，真の陽性例に対して相対的に偽陽性例が増加することに留意する必要がある<sup>6)</sup>。

## ● HTLV-1 キャリア妊婦の管理

### ■検査時期と告知

長崎県では，HTLV-1 検査は，妊婦が精神的に不安定な妊娠初期・中期を避けて，妊娠 29～32 週頃に行っている。そして，妊娠 35 週頃に最終判定の結果を産科医から妊婦へ告知し，HTLV-1 キャリア妊婦には栄養方法の選択などについて説明している（図 2）<sup>2,3)</sup>。一方，児の HTLV-1 抗体検査については，① HTLV-1 キャリア妊婦から産まれた新生児は母親からの移行抗体のためほぼ全例が HTLV-1 抗体陽性であること，② 移行抗体は生後 3～6 か月で陰性化するこ

と，③ 児の抗体陽転は 3 歳までに起こることを考慮して，退院時に産科医から 3 歳を過ぎてから長崎県内の小児科医療機関を受診して検査を受けるように説明している（図 2）<sup>2,3)</sup>。

告知に際しては，妊婦のプライバシー保護のため，① HTLV-1 キャリアのリストをつくらないこと，② 医療スタッフは妊婦が HTLV-1 キャリアであることを家族内の誰と誰が知っているのかを把握すること，③ 担当医以外は HTLV-1 抗体陽性の結果説明をしないことなどの対策が必要である。また，キャリアの告知には，夫婦の家庭内問題などさまざまな問題が生じうるため，相談・カウンセリング体制の確立も重要であり，キャリア妊婦とその家族が HTLV-1 感染症に関するさまざまな疑問，悩み，不安などの問題を抱えたときに，相談を受け入れてもらえるシステムを用意することが大切である（図 3）<sup>2,3)</sup>。長崎県では

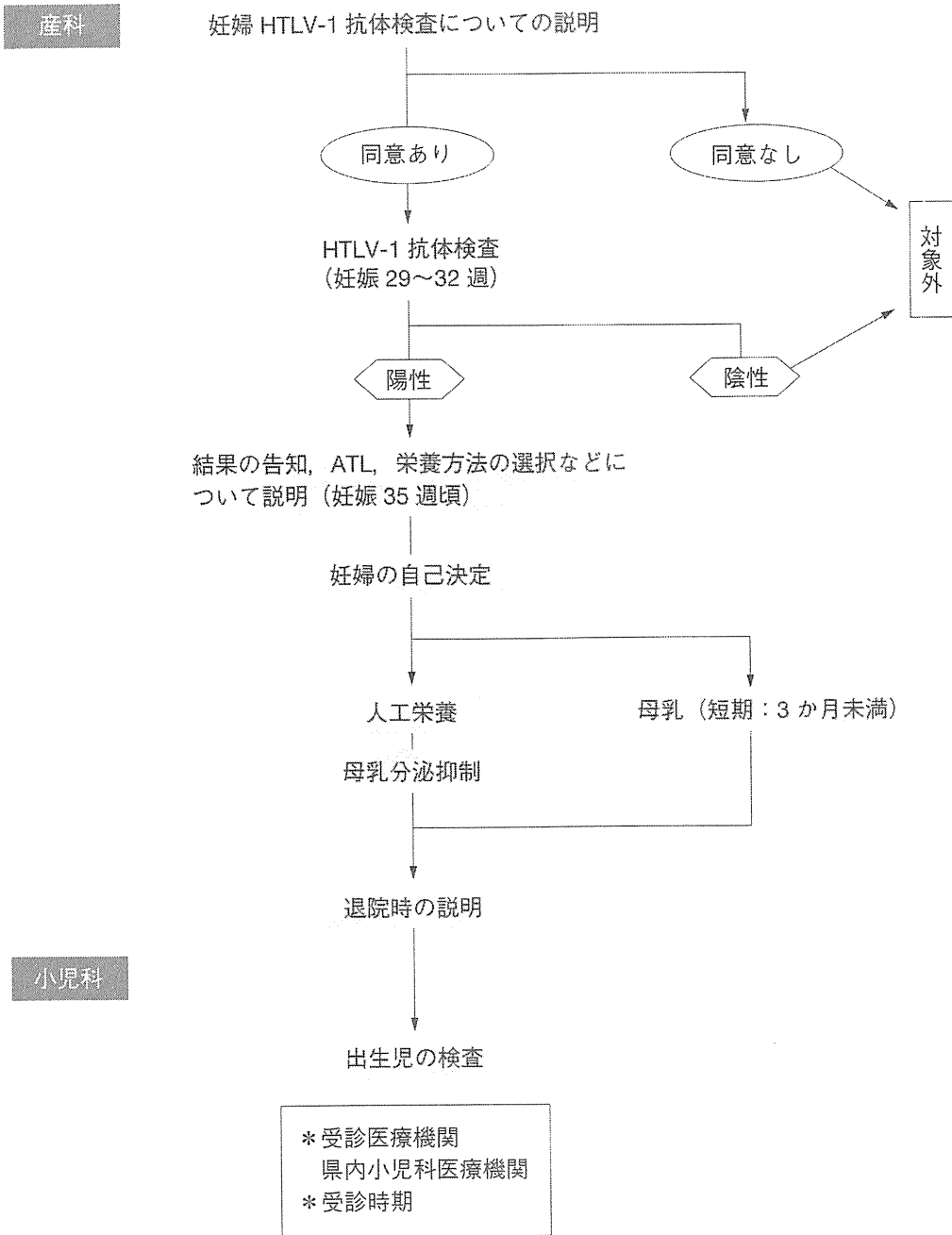


図2 長崎県 ATL ウイルス母子感染防止研究協力事業プログラムチャート (文献2より)

原則として担当した産科、小児科医師が対応しているが、問題が多いケースの医学的事項については長崎県 ATL ウイルス母子感染防止研究協力事業連絡協議会が対応し、家庭環境やノイローゼなどの問題については保健師あるいは嘱託精神科医が対応するシステムを構築している (図3)。また、対応がきわめて難しいケースについては、長崎大学産婦人科教授もしくは小児科教授が対応している。

### ■栄養法の選択

母乳栄養と人工栄養の優劣は、状況に応じてどちらの栄養法が児にとってメリットが大きくなるのかを考えなくてはならない。母乳栄養はビタミン K、ビタミン D や鉄分は不足しがちであるが、免疫学的、栄養学的に人工栄養より優れているところはたくさんあり、母子関係の形成に重要な役割を果たしていると考えられる。最終的に栄養法を選択は親の意思によって決定されるが、キャリア妊婦がいずれの栄養法を選択した場合でも医療

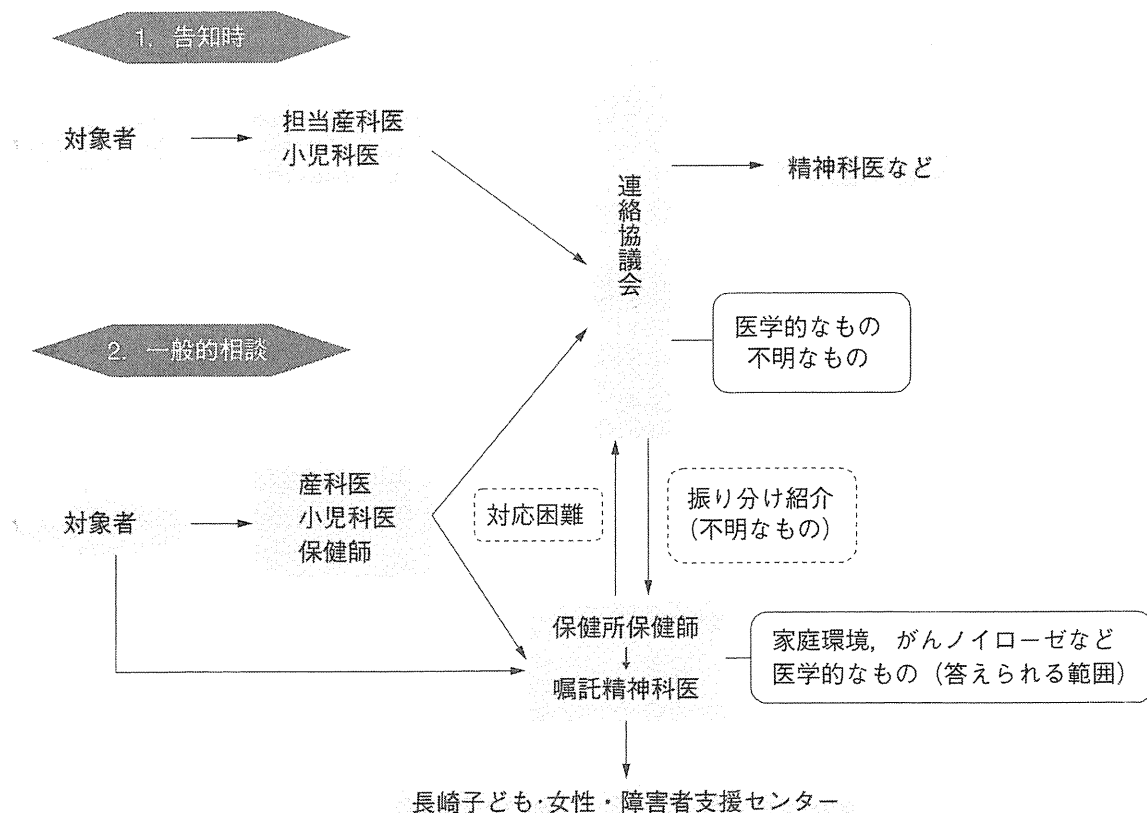


図3 相談・カウンセリング体制（長崎方式）（文献2より）

スタッフは妊婦の選択を尊重することが重要である。しかし、いったん発症すると予後不良なATLの最も有効な予防法は、母乳を介したHTLV-1の母子感染を予防することである。妊婦のHTLV-1抗体を検査することで、HTLV-1抗体陰性の妊婦は安心して母乳保育を行い、キャリアと診断された妊婦は自分自身の意志で人工栄養を選択し、児へのHTLV-1母子感染のリスクを低下させることができる。HTLV-1母子感染の予防効果からすると人工栄養が最も推奨される栄養法であり、長崎県ではHTLV-1キャリアと確定診断された妊婦のほとんどが人工栄養を選択している（表3）<sup>2,3)</sup>。

●人工栄養

HTLV-1の母子感染予防効果の最も高い栄養法であり、母子感染率は母乳栄養を選択した場合の20.5%から2.4%に低下する（表1）<sup>2,3)</sup>。母子関係の形成は直接に母乳を与えるだけで強くなるわけではないので、母乳を与えられなくても抱っこして、目を見つめ、語りかけ、児とふれあう時間をつくるなど母親が児に普通にかかわるこ

とで母子間の愛情は自然に形成される<sup>2,3)</sup>。また、人工栄養は感染症、アレルギー、乳児突然死症候群（SIDS）のリスクになりうる可能性も指摘されているが、それぞれ人混みを避ける、離乳を急がない、うつ伏せ寝や喫煙を避けるなどの一般的な注意点を守ることによってリスクを軽減することができる<sup>2,3)</sup>。HTLV-1に感染することは、生まれてくる児にとってはきわめて重大な問題であり、母子感染を予防するために、母乳栄養の重要性を理解したうえで親の意志で人工栄養を選択することは、親の愛情として尊重されるべきであろう<sup>2)</sup>。

●短期母乳栄養（3か月未満）

3か月未満の短期母乳保育を選択した場合の母子感染率は、人工栄養ほどではないが、母乳栄養を選択した場合の20.5%から8.3%以下に低下する（表1）<sup>1,2)</sup>。短期母乳栄養が母子感染率を低下させるメカニズムは解明されていない<sup>2,3)</sup>。したがって、短期母乳栄養を推奨しているわけではなく、母乳を飲ませることができて、かつ母子感染率もある程度下げることができる短期母乳栄養は次善の策と考えている<sup>2,3)</sup>。また、3か月で授

妊婦

表3 長崎県のHTLV-1キャリア妊婦1,178例が選択した栄養方法の動向：1999～2010年  
(文献3より引用, 改変)

年度	人工栄養	混合栄養	短期母乳栄養	長期母乳栄養	合計
1999	117 (79.1)	5 (3.4)	8 (5.4)	18 (12.2)	148
2000	97 (77.0)	2 (1.6)	15 (11.9)	12 (9.5)	126
2001	85 (73.3)	3 (2.6)	15 (12.9)	13 (11.2)	116
2002	70 (66.0)	2 (1.9)	18 (17.0)	16 (15.1)	106
2003	76 (73.1)	2 (1.9)	10 (9.6)	16 (15.4)	104
2004	63 (55.3)	7 (6.1)	18 (15.8)	26 (22.8)	114
2005	53 (72.6)	1 (1.4)	11 (15.1)	8 (11.0)	73
2006	43 (61.4)	1 (1.4)	18 (25.7)	8 (11.4)	70
2007	43 (62.3)	0 (0.0)	13 (18.8)	13 (18.8)	69
2008	38 (59.4)	0 (0.0)	15 (23.4)	11 (17.2)	64
2009	56 (64.4)	1 (1.1)	22 (25.3)	8 (9.2)	87
2010	70 (69.3)	1 (1.0)	21 (20.8)	9 (8.9)	101
合計	811 (68.8)	25 (2.1)	184 (15.6)	158 (13.4)	1,178

( ) : %

乳を中止して人工栄養に切り替えるためには、2か月くらいから授乳中止の方法についてキャリアの母親が助産師、保健師、あるいは看護師に相談できるような体制が必要である<sup>2)</sup>。

#### ●凍結母乳栄養

母乳を搾乳し、家庭用の冷凍庫で24時間以上凍結したのち、解凍した母乳を哺乳瓶で児に与える方法である<sup>2,3)</sup>。母乳中のHTLV-1感染リンパ球を含むリンパ球は不活化されるが、それ以外の母乳成分は児に移行する。直接授乳できないことは人工栄養と変わりはない。

#### ●長期母乳栄養法

母乳栄養を6か月以上継続したときの母子感染率は20.5%である(表1)<sup>2,3)</sup>。

#### ■長崎県での母乳回避介入試験の効果

長崎県では、妊婦のHTLV-1抗体陽性率は1988年の時点で7.2%であったが、2003年には2.0%以下になり、2010年には1.2%にまで低下している(表2)<sup>2,3,5)</sup>。これは、女性のライフスタイルの変化に伴う人工栄養や短期母乳の普及を反映しているのかもしれないが、2010年のHTLV-1キャリア妊婦の陽性率を出生年代別にみると、1987年に介入試験が始まったが、1985年以前に出生した妊婦のHTLV-1抗体陽性率は1.26% (109/8,655例)であるのに対して、1986年以降に出生した妊婦のそれは0.74% (10/1,343

例)であった(表4)<sup>5)</sup>。最終的な結論を得るには、さらに数年間の継続調査が必要であるが、介入以降に出生した妊婦に限った長崎県のHTLV-1キャリア率は、キャリア率が低頻度の関東や関西地域とほぼ同レベルにまで低下しており、現在のところ母乳回避介入はHTLV-1母子感染予防とATL撲滅に対して有効であると考えている<sup>7)</sup>。

#### ●講習会や市民公開講座を通じた

#### ●啓発活動の重要性

表3に長崎県のHTLV-1キャリア妊婦1,178例が選択した栄養方法の動向を示す<sup>2,3,5)</sup>。その結果、人工栄養を選択した妊婦は811例(68.8%)で最も多く、次いで短期母乳栄養の184例(15.6%)、長期母乳栄養の158例(13.4%)、人工栄養と母乳栄養を併用している混合栄養の25例(2.1%)であった。キャリア妊婦が選択した栄養法の経年推移をみると、1999年は79.1%であった人工栄養の選択率が、2008年には59.4%にまで落ち込んだが、2009年に64.4%、2010年には69.3%とV字回復の傾向にある。これは、2008年以降にHTLV-1キャリアとATLを話題に取り上げた市民公開講座を開催した効果と思われる。講習会では、血液内科医がATL治療の現状、HAM患者の会代表による患者の視点、産婦



表4 出生年代別にみた HTLV-1 キャリア妊婦陽性率：2010年度（文献5より引用，改変）

年代別	1次検査		2次検査		陽性率 (%)	陽性数/一次検査実施数 (%)
	実施数	対象数	対象数	陽性数		
1956～1960	0	0	0	0	0.00	109/8,655 (1.26%)*
1961～1965	10	0	0	0	0.00	
1966～1970	312	5	5	4	1.28	
1971～1975	1,938	30	30	26	1.34	
1976～1980	3,372	57	57	46	1.36	
1981～1985	3,023	41	41	33	1.09	
1986～1990	1,239	12	12	10	0.81	10/1,343 (0.74%)**
1991～1995	104	0	0	0	0.00	
合計	9,998	145	145	119	1.19	

\*：妊婦が母乳回避介入試験の開始以前に出生した世代，\*\*：妊婦が母乳回避介入試験の開始後に出生した世代

人科医と小児科医が HTLV-1 母子感染予防について講演を行っている。定期的な講習会や市民公開講座の開催は、医療者側と市民の双方にとって HTLV-1 母子感染に対する意識を高めることにつながり、HTLV-1 母子感染予防システムの確立にきわめて重要であることを最後に強調しておきたい。

## ● おわりに

HTLV-1 母子感染の主要経路は母乳感染である。妊婦の HTLV-1 抗体スクリーニングでキャリアと診断された妊婦が人工栄養を選択することによって、母子感染は母乳栄養を選択した場合の 20.5% から 2.4% にまで減少させることができる。出生後の栄養法の選択は、児にとっては将来の ATL をはじめとする HTLV-1 感染に関連した難治性疾患の発症リスクを最も効果的に回避しうる最初で最後のチャンスであることに十分に留意して、医療者（保健師，助産師，看護師，医師）は保健指導を行う必要がある。また、妊婦の

HTLV-1 抗体スクリーニング検査を開始するに際しては、結果の告知とその後のフォローが行える相談・カウンセリング体制を確立しておくことが重要である。

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# Heterogeneity in clonal nature in the smoldering subtype of adult T-cell leukemia: continuity from carrier status to smoldering ATL

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**Abstract** To better understand indeterminate HTLV-1 carriers and smoldering (SM) subtype of adult T-cell leukemia (ATL), HTLV-1 proviral integrated status, proviral load (PVL) and ATL-related biomarkers were examined in 57 smoldering cases, including unusual carriers with a percentage of ATL-like cells. We found that according to Southern blot hybridization analytic features, 28 patients with SM ATL could be divided into 3 groups consisting of 16 (57.4%) patients with a monoclonal band, 6 (21.4%) with oligoclonal bands and the remaining 6 with smears. Although no clinical differences were observed among the 3 SM subtypes, HTLV-1-infected CD4 T-cell counts increased in order of poly-, oligo- and monoclonal subtypes. This trend began in the carrier stage and also was observed in PVL, CD25 and CCR4, indicating that a clone consisting of leukemic phenotypic cells was continuously growing. Moreover, the antigen modulation rates of CD26 and CD7 and the increasing rate of CD25 and CCR4 cells were closely correlated to growing clonal size, indicating

that these markers had the possibility to predict a monoclonal band. In particular, CD26 or the ratio of CD26/CD25 had a validity differential for leukemic nature and predictive detection of clonal band. Conclusively, the present study shows that smoldering ATL is heterogeneous in the leukemogenic process, and the behavior of CD26 plays a central role in the evolution from early occult to overt smoldering ATL.

**Keywords** ATL · HTLV-1 · Provirus · Southern blot · Leukemogenesis

## Abbreviations

HTLV-1	Human T-cell leukemia virus type-1
ATL	Adult T-cell leukemia
SBH	Southern blotting hybridization
PMNC	Peripheral blood mononuclear cell
LDH	Lactate dehydrogenase
sIL-2R	Soluble-interleukin-2 receptor

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## Introduction

Human T-cell leukemia virus type 1 (HTLV-1) was found to be a causative retrovirus of a T-cell malignancy, designated as adult T-cell leukemia (ATL) [1, 2]. All ATL cells, without exception, carry the HTLV-1 provirus in the same genomic site in each case, indicating that provirus insertion is an excellent biomarker for the cellular clonality of ATL and the integrated provirus can be used as the proviral load (PVL) [3] to estimate viral and cellular burden. Proof of clonality is essential for a diagnosis of malignant neoplasm, but it should be noted that HTLV-1-infected cells also are clonally expanded to maintain

persistent infection [2, 4]. Thus, changes in the abundance of HTLV-1-infected cell clones play an important role in persistent infection and ATL leukemogenesis. The clonality of provirus-carrying cells is usually demonstrated by Southern blot hybridization (SBH). However, since the detection sensitivity of this assay is limited (about 5%) [5, 6], it is unavailable for samples including small clones with 5% or fewer monoclonal cell populations.

Recently, we have had many opportunities to see patients with smoldering ATL and unusual carriers with high HTLV-1 PVL or with a proportion of ATL-like cells. We sometimes struggle to distinguish such borderline cases between carriers and smoldering ATL. ATL cells phenotypically resemble Treg cells expressing CD4, CCR4 and CD25. On the other hand, ATL cells aberrantly express 100 or more cell surface receptors and ligands [7, 8]. Such aberrantly expressed receptors consist mainly of natural, adaptive and ectopic types, some of which are considered to be involved in leukemogenesis [9]. In particular, down-regulation of CD3, CD7 and CD26 are observed during the early phase of leukemogenesis [9]. However, little is known about the behavior of cells concurrently expressing CD4, CD25, CCR4 and CD26 in the carrier to SM stages using in vivo practical samples. Accordingly, to better understand indeterminate carriers and smoldering ATL, the present study was focused on the implication between the SBH features reflecting clone size and cellular changes in phenotype and number. In particular, CD26 is noted to be one of the prodromal cellular changes, because the down-regulation of CD26 begins in the carrier stage and persists continuously till the completion of ATL.

## Materials and methods

White blood cell counts, morphological data, serum lactate dehydrogenase (LDH) activity and soluble interleukin-2 receptor (sIL-2R) were used from routine laboratory data. Peripheral blood samples were collected from our ATL and HTLV-1 carrier clinic, consisting of 28 patients with smoldering ATL carrying 5% or more ATL-like cells in blood, 12 unusual carriers with around 5% ATL-like cells, and 17 common (healthy) carriers. Thirty-four samples from patients with leukemic chronic or acute ATLs were used as a positive control. Morphological evaluation was microscopically conducted by hematological specialists.

High-molecular-weight DNA was extracted from peripheral blood mononuclear cells (PMNC) using a QIAmp DNA Blood Mini Kit (Qiagen GmbH, Hilden Germany). PVL was quantified by LightCycler Technology (Roche Diagnostic K.K., Tokyo, Japan) using hydro-probes and previously described primers [10–12]. Normalization was done using the  $\beta$ -globin gene and the PVL was

expressed as copy number per  $10^4$  cells or percent for PMNC. This study was done under the approval of our institutional board.

## Clone assay of SBH

SBH analysis was performed by a method described previously with modification, using 7 mixtures of probes covering the total region of the digoxigeninated provirus and the restriction enzymes of EcoR-1 and Pst-1 [13, 14]. Pst-1 cuts 4 sites of the provirus, but EcoR-1 cannot cut within the provirus. Therefore, to determine clonally related sharp band or polyclonally related smear bands, EcoR-1 digestive genomic fragments were used. To assay clonality accurately, we monitored 1.5, 3 and 5% clonal cell controls every time. Band patterns were estimated using a densitometer (Fujifilm Life Science, Science Lab 2005, and Tokyo, Japan).

## Flow cytometry analysis for cell surface antigens

The positive rate for CD3, CD4, CD7, CD25, CD26 and CCR4 was measured by a routine method using whole blood according to the manufacturer's instructions (BD FACSCato-II, Nixon BD, Inc, Tokyo, Japan). The association between CD7 and CD26 antigen modulation and the positivity of CD4, CD25 and CCR4 cells was assessed by using the four-color staining method according to the manufacturer's instructions. Co-expression with CD4, CD25, CCR4 and CD26 was assessed by a four-color flow cytometric method using CD26-FITC, CD4-PerCP, CD25-APC and CCR4-PE. The rate (%) of CD4<sup>(+)</sup>CD25<sup>(+)</sup>CCR4<sup>(+)</sup> cells relative to all CD4 cells and the co-expression rate of CD26 with CD4<sup>(+)</sup>CD25<sup>(+)</sup>CCR4<sup>(+)</sup> cells was measured according to the BD FACSCato-II protocol (BD, Inc., Tokyo, Japan).

## Statistical analysis

Data are expressed mainly as the median and analyzed using the Mann-Whitney test. *P* value of below 0.05 was considered to be statistically significant. Analyses were performed with Stat Flex version 6.5 software packages (Artech Inc., Osaka, Japan).

## Results

Classification features of band patterns using a densitometer

The band patterns in SBH analysis using EcoR-1-digestive genomic fragments were mainly estimated by a densitometer graph. The densitometer graphs equivalent to band

status are classified into five patterns (Fig. 1a): patterns-1 and -2 are light and dense smear bands with no difference in staining density; pattern-3 is a hill ridge, irregular shape with vertical stripes or a low and broad projection with smear bands; pattern-4 is a low/sharp peak type; and pattern-5 is a sharp high peak. In vivo clonal status of the five patterns based on SBH features, as shown in Fig. 1b, is considered to correspond to few small clones in pattern (P)-1, many small clones with few growing clones in P-2, oligoclonal of a detection limit in P-3, and clearly detectable monoclonal(s) with various background clonal status in P-4 and 5.

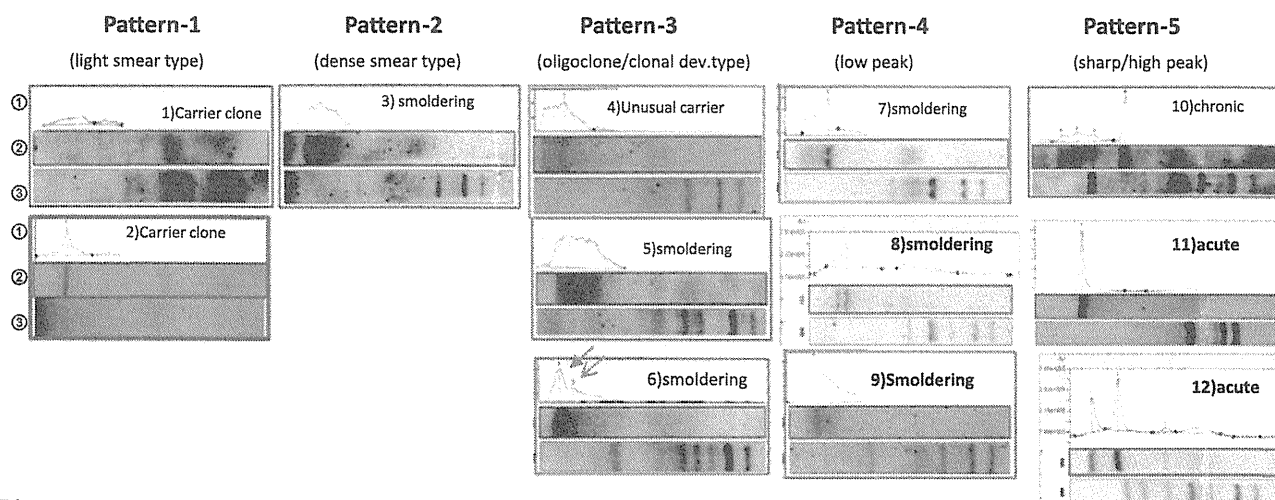
SBH analysis allocated 57 cases to 17 of P-1, 11 of P-2, 12 of P-3, 6 of P-4 and 11 of P-5. The relations between intra- or inter-disease status and the band patterns are summarized in Table 1. Consequently, smoldering ATL was the most heterogeneous for SBH patterns; 16 (57.1%) of 28 smoldering ATLs were P-4 and -5 (large clone consisting of at least 5% monoclonal cells), while the remaining 12 (42.9%) were P-1, -2 and -3 (equivalent to polyclonal or oligoclonal band). Actually, Fig. 2 is an interesting example of an SM subtype showing dense

smear bands and abnormal cells with an aberrant phenotype of 73% CD4, 77% CD25 and 21% CD26.

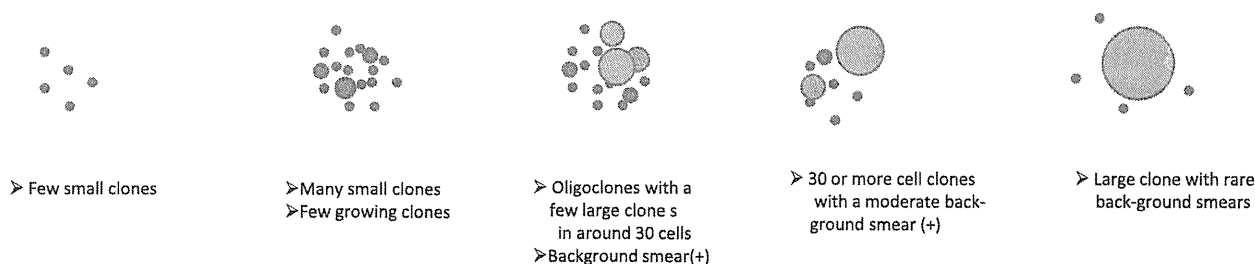
Cyto-oncological characteristics of the three SM subtypes

To characterize ATL-related biomarkers in the three subtypes of SM, the findings were comprehensively compared with those of healthy carriers, unusual carriers and patients with chronic ATL (Table 2). First, clonal expansion-associated biomarkers, such as PVL, HTLV-1-infected CD4 T-cell counts in 1 μL peripheral blood and the serum level of sIL-2R increased regularly in the order of poly-, oligo- and monoclonal SM subtypes. Figure 3 shows the line graphs on increasing fold (rate) of PVL and total lymphocyte, all CD4 T-cell and HTLV-1-infected CD4 T-cell counts converted from Table 2. The graph shows two distinctive patterns (solid lines of PVL and infected CD4 T-cell vs. broken lines of total lymphocyte and all CD4 T-cell). In contrast to the horizontal part of the broken lines, the solid lines are gradually elevated, meaning that the provirus-carrying CD4 T-cells gradually increase

(A) Pattern Classification of SBH features



(B) The images of in vivo clonal status in each Pattern



**Fig. 1 a** The densitometry patterns for HTLV-1 proviral integration status according to SBH band features using a restrictive enzyme of Eco-R1 and representative cases. Subjects were mainly classified into five patterns according to densitometer images. ① Densitometry

graph. ② SBH analysis for Eco-R1-digestive genomic fragments. ③ SBH analysis for Pst-1-digestive genomic fragments. **b** The image of in vivo clonal status in each pattern

**Table 1** The pattern distribution of SBH features in intra- and inter-diseases

	Smear band		Oligoclonal bands P-3	Monoclonal bands		Total
	P-1	P-2		P-4	P-5	
Common carriers	11	5	0	0	(1) <sup>a</sup>	17
Unusual carriers	5	1	6	0	0	12
Smoldering ATL	1	5	6	6	10	28

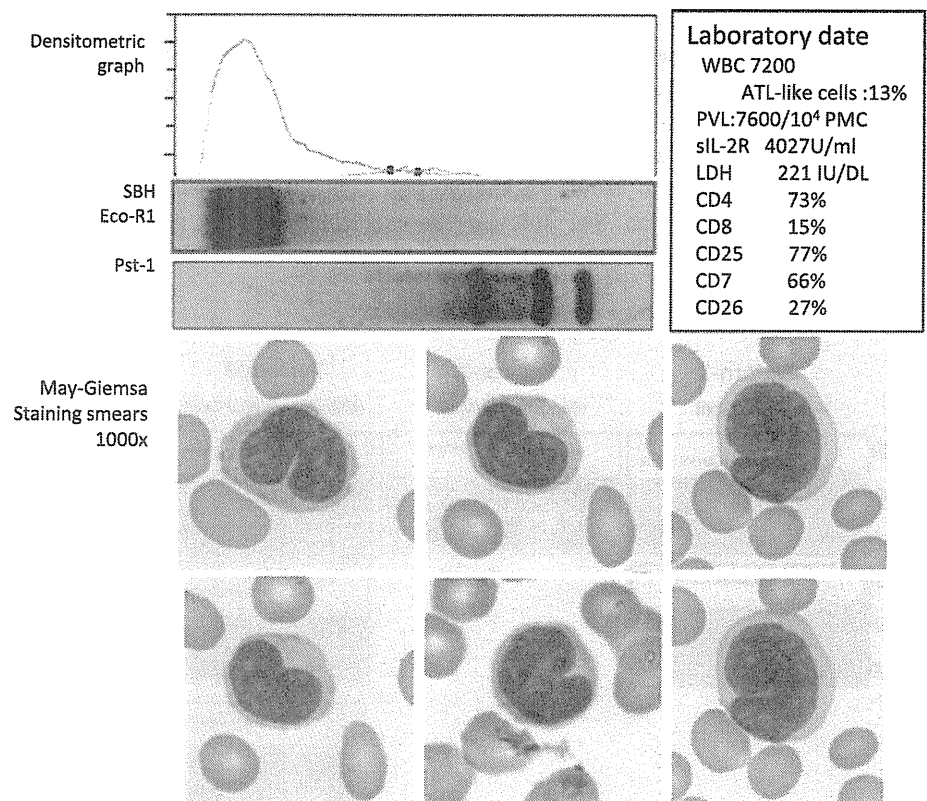
The criteria of the classification is explained in the text. P-1, P-2, P-3, and P-4 and P-5 generally correspond to light smears, dense smears, oligoclonal bands and monoclonal bands, respectively. Of leukemic type ATL, including the chronic and acute type, SBH features in smoldering ATL were the most heterogeneous

*Common carriers* HTLV-1-seropositive individuals without any HTLV-1-associated disorders

*Unusual carriers* those who have clinico-cytological findings similar to that of the smoldering subtype of ATL

<sup>a</sup> Carrier clone

**Fig. 2** A representative case presentation of the polyclonal smoldering subtype (SM) showing a polyclonal dense smear in SBH analysis and the smoldering subtype with full hematological criteria



regardless of the almost stable counts of non-infected CD4 T-cells during the entire period of smoldering ATL.

The positive values of CD4, CD25, CCR4, CD7 and CD26 subsets (%) were observed to change continuously and concurrently in the order of common carriers, unusual carriers, polyclonal SM, oligoclonal SM and monoclonal SM. In order to interpret these data in detail, a line graph was used (Fig. 4). CCR4 and CD25 cells increased concurrently and sharply from the common carrier stage to the oligoclonal stage. The down-regulation of CD26 was initiated in the unusual carrier stage and kept falling continuously by chronic stage. The fluctuations of CD4 and

CD26 showed an opposing trend, and the interval between CD4 and CD26 (solid triangle and gray circle) gradually enlarged with the increasing cell number of 32, 54, 115 and 163 cells. Such behavior of CD26 was expected to play a central role in budding of ATL in the early stage of multi-step leukemogenesis.

#### Clinico-oncological usefulness of CD26

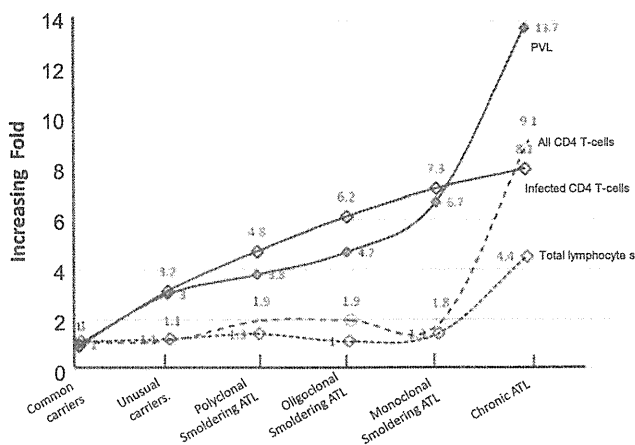
From the results described above, CD26 appears to be closely associated with the evolution of SM. In contrast to characteristic phenotypes in overt ATL cells, those of

**Table 2** Comparison of the measurement value (mean) of ATL-related biomarkers among the polyclonal, oligoclonal and monoclonal SM subtypes

	Carrier stage		Smoldering stage			Chronic
	Common	Unusual	Polyclonal	Oligoclonal	Monoclonal	
PVL (%)	5.9	17.9	22.6*	28.0	39.3*	78.3
Total Ly counts	1750	1950	2308	1732	2306	7659
All CD4 T-cell	786	882	1570	1513	1377	7220
Infected CD4	102	330	480*	628	744*	8346
LDH (IU/mL)	199	200	222	186	179	257
sIL-2R (U/mL)	868	765	1425	1877	1887	6106
CD4 (%)	43	48	52	55	56	79
CD25 (%)	14	22	32	45	44	75
CCR4 (%)	14	25	38*	50	58**	76
CD7 (%)	63	65	70	47	50	11
CD26 (%)	43	41	41*	28	22**	7

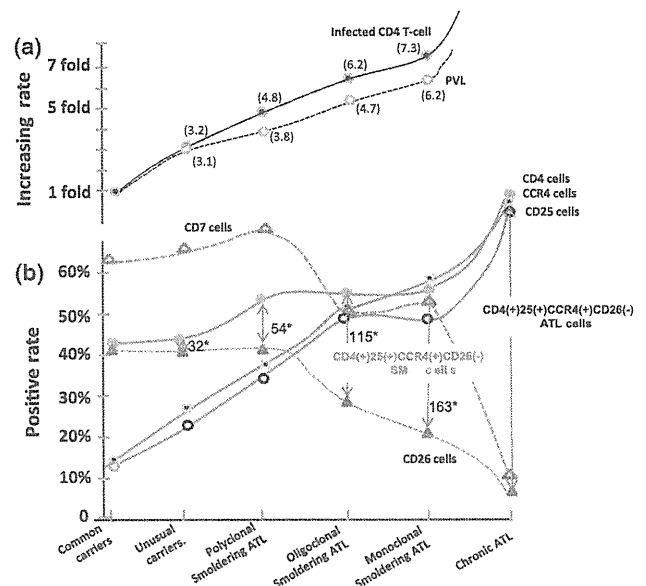
Statistically significant ( $P < 0.05$ ) between \* and \*\* in PVL, infected CD4 cell number, CCR4 (%) and CD26 (%)

HTLV-1-infected CD4 T-cell number/1  $\mu$ L p-blood = total Ly counts  $\times$  CD4%/100  $\times$  PVL (%/100)



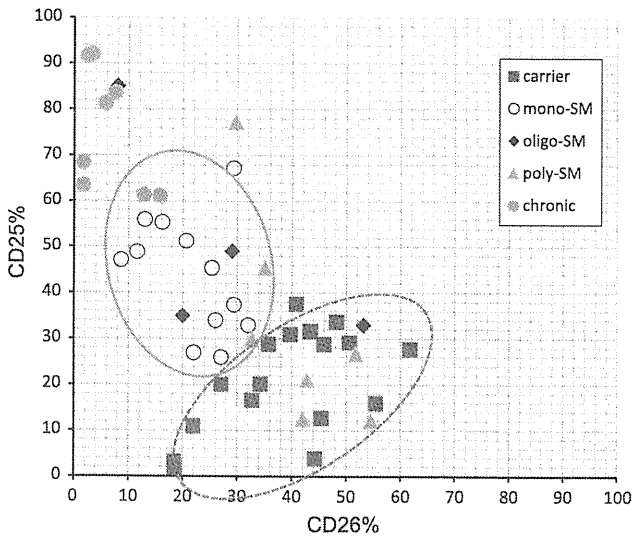
**Fig. 3** The difference in the line-graph patterns between HTLV-1-infected and uninfected cells. PVL and infected CD4 T-cell counts gradually increased from the early carrier stage to the last stage of smoldering, while the HTLV-1-uninfected cell population was stable, indicating that the discrepancy was explained by the infected leukemic clonal expansion alone

occult ATL (SM) cells are now controversial. One of the reasons for this is thought to be the difficulty in identifying SM cells. Therefore, to overcome these problematic issues, a dot-plot graph for CD26 versus CD25 and a four-color staining method were applied. The dots of CD26 and CD25 were mainly clustered into two areas (Fig. 5): 11 of monoclonal SM were clustered into a solid line circle, while oligoclonal and polyclonal SM were widely distributed. On the other hand, carriers were compactly clustered within the broken line circle. This indicates that also the 3 SM subtypes are not always homogeneous in biological character. Since the CD25 versus CD26 dot graph only



**Fig. 4** The rate of change in each ATL-related biomarker. There were two major types of curves; a concurrent increasing type with tumor burden and a decreasing type with aberrant down-regulation. **a** The increasing rate (fold) relative to 102 HTLV-1-infected CD4 T-cells in 1  $\mu$ L of blood. **b** Comparison of the positive rates in each disease state equivalent to P-1 (common carriers), P-2 (dense smears), P-3 (oligoclonal), and P-4 and P-5 corresponding to the monoclonal phase. Asterisks represent predicted CD4<sup>(+)</sup>CD26<sup>(-)</sup> cell number equivalent to a major clonal expansion representing the absolute increased tumor burden

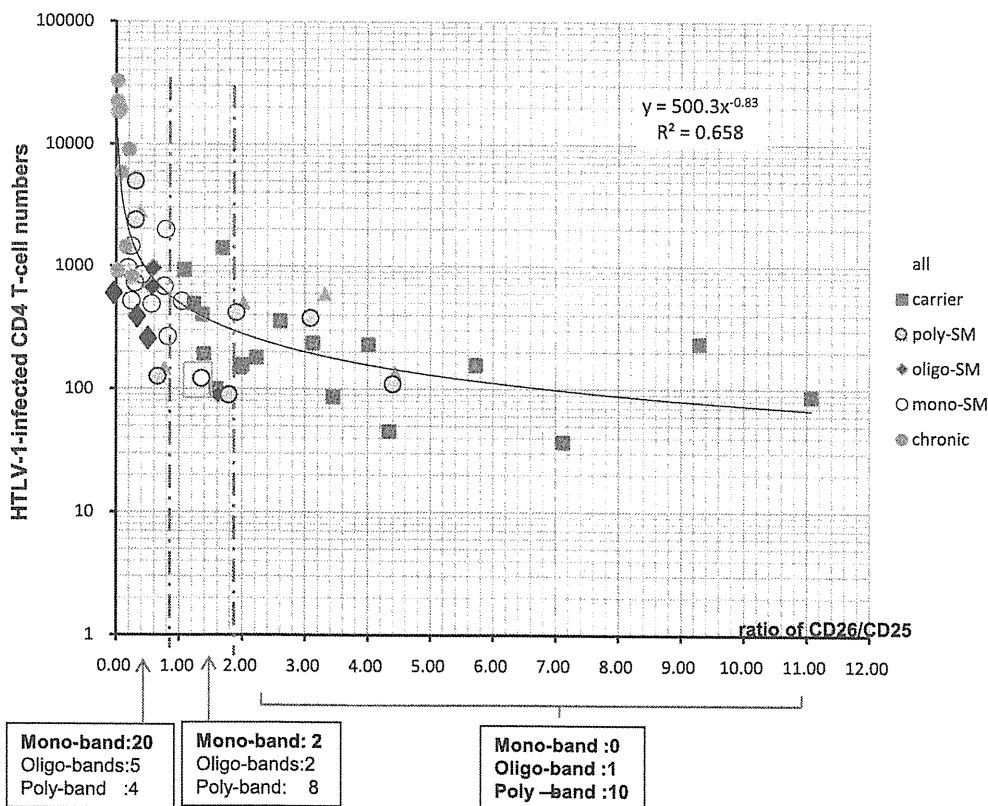
hinted at the heterogeneity of SM, we examined the clinico-oncological role of CD26 using 3 parameters of HTLV-1-infected cell counts, the CD26/CD25 ratio and SBH features. As shown in Fig. 6, the number of HTLV-1-infected CD4 T-cells was closely correlated to the ratio of



**Fig. 5** CD26 versus CD25 twin dot graph, showing that there were two hot areas. The cells clustering in the same area have uniform bio-characteristics. Monoclonal SM was concentrated in the same area, but polyclonal SM and carriers were distributed sparsely and widely. Red squares within dotted line circle 3SM subtypes were scattered into both circles

CD26/CD25 ( $R^2 = 0.6586$ ), and the clustering patterns were characteristic.

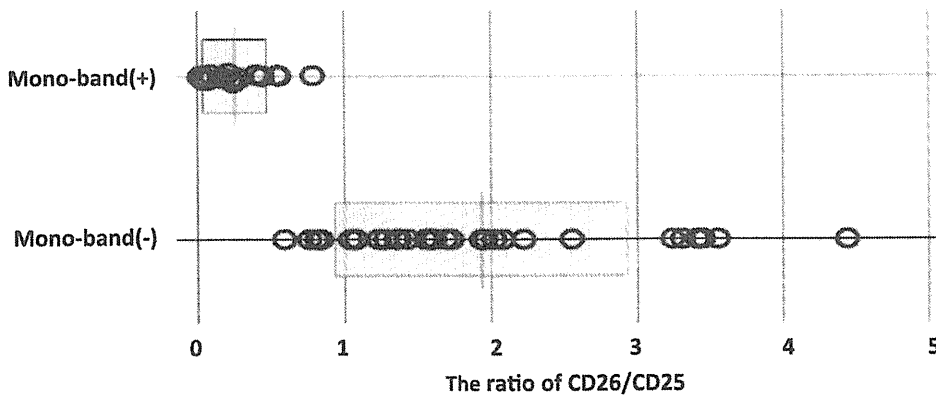
Samples with monoclonal band were mainly clustered in a high area within 0 to <1 of the X-axis. Most other samples were widely distributed in an area of around 1.00–11.00 of the Y-axis. Thus, the CD26/CD25 ratio represents the degree of advance in the leukemic process, comparable to the growing level of an ATL cell clone. Actually, this was demonstrated to be an indicator of a monoclonal band using a distribution graph and a receiver operating curve (ROC). That is, as shown in Fig. 7a, the ratios of CD26/CD25 were clearly separated by the presence or absence of a monoclonal band. The ROC analysis gave a high area under the curve (AUC) of 0.90, and sensitivity, specificity, and positive and negative predictive values were 87.0, 83.0, 80.0 and 89.0%, respectively (Fig. 7b), when the ratio of cutoff value (COV) was 1.04. The detective test performances of CD26 and CD7 alone for a monoclonal band were 0.82 and 0.81 AUC, respectively. This simple predictive method as an alternative to the SBH test, which is time- and labor-consuming, may be practically useful.



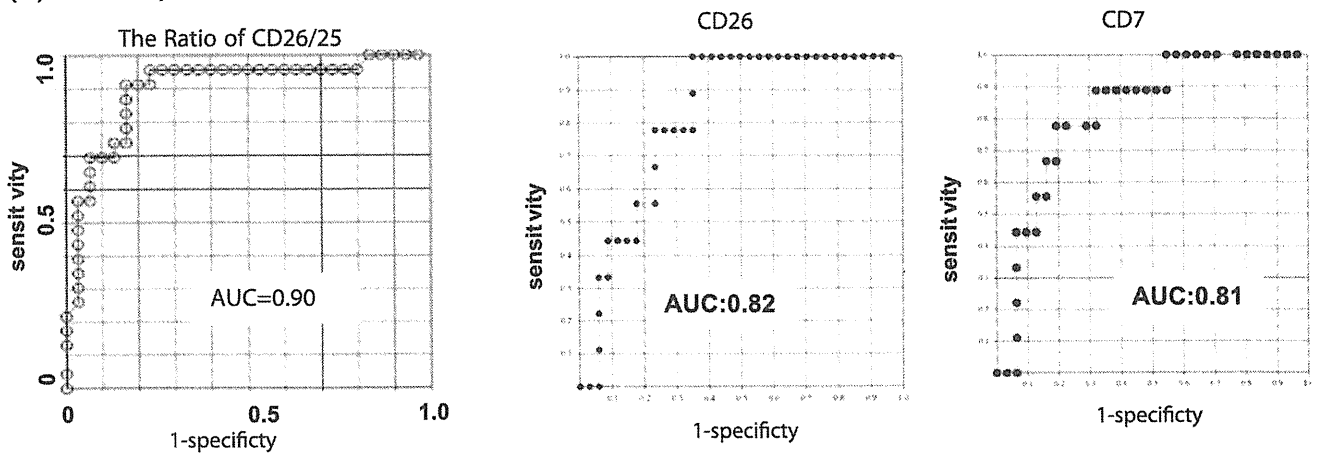
**Fig. 6** Three parameter “twin dot-plot graph” between HTLV-1-infected CD4 T-cell numbers and the carrier or disease subtypes. Samples with the same band pattern showed a tendency to gather in the same areas bordered by the CD25/

26 ratio lines, such as most samples with monoclonal band (mono-band) in an area within 0.00–1.00 of the X-axis, and most samples with smears (poly-band) in an area within 1.00–11.00 of the X-axis

(A) Distribution graph



(B) ROCAnalysis



**Fig. 7** a The distribution graph of each sample with or without a monoclonal band. The median values of mono-band (+) or (-) were 0.32 and 1.98. b The receiver operating curve (ROC) analysis

Correlation between down-regulation of CD26 antigens and SBH features

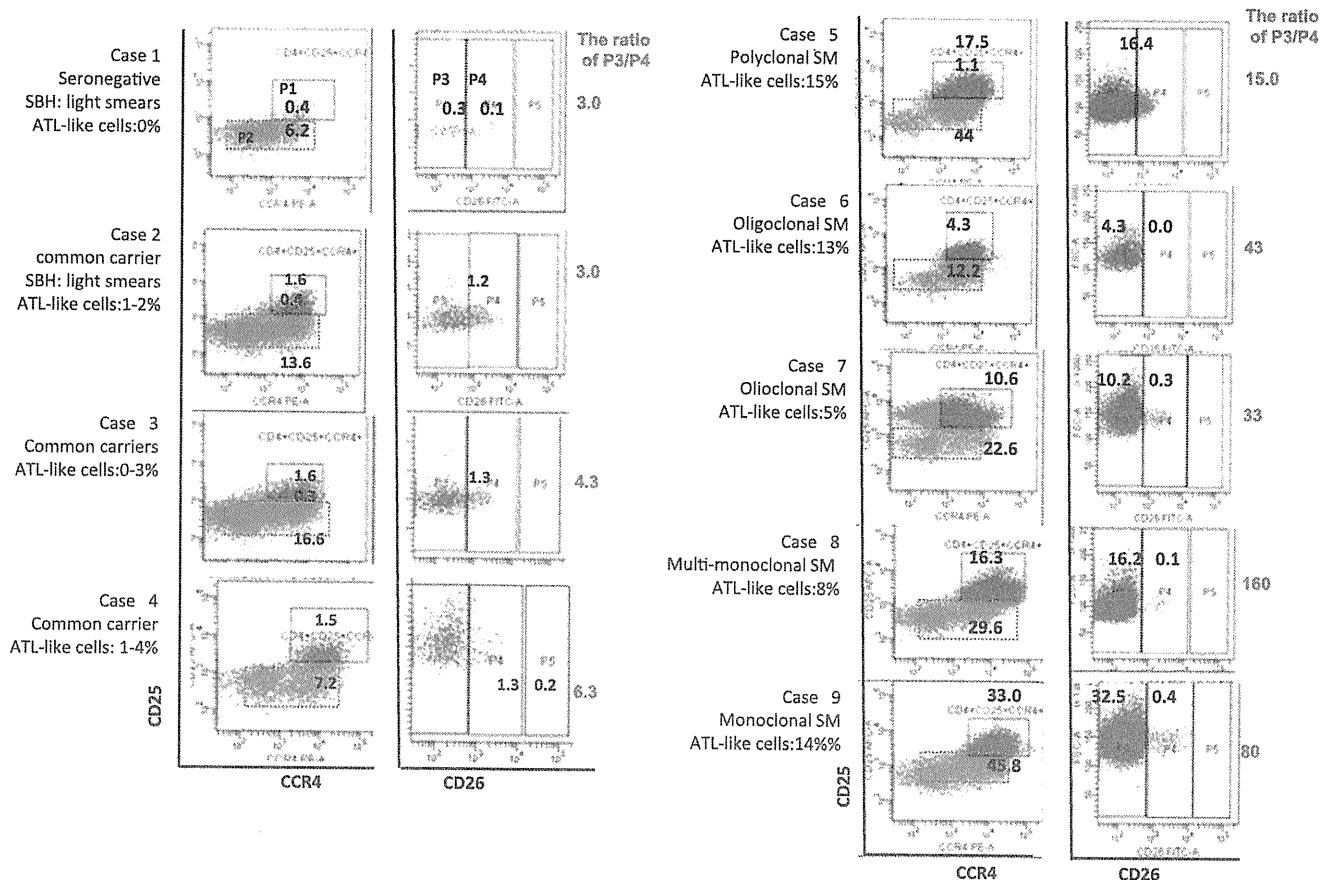
It is known that CD7 and CD26 antigens are lost in chronic and acute ATL cells. The present study revealed that the loss of CD26 antigens was initiated early in the pattern-2 or -3 stages. To confirm whether CD4<sup>(+)</sup> CD25<sup>(+)</sup> CCR4<sup>(+)</sup> cells were concurrently expressed, a four-color staining flow cytometric method for CD4, CD25, CCR4 and CD26 was used (Fig. 8). CD4<sup>(+)</sup> CD25<sup>(+)</sup> CCR4<sup>(+)</sup> cells (P1 square) were 1% or less, of which 75% (0.3% of total CD4 cells) were CD26 negative and 25% were CD26 positive in a healthy individual seronegative for HTLV-1 (case 1). That is, the CD25<sup>(-)</sup>/CD26<sup>(+)</sup> ratio was 3.0. On the other hand, the ratio in common carriers and SM subtypes was about 3.0–10.0 (cases 2–4) and 10 or more (cases 5–9), respectively. This phenomenon regarding the loss of CD26 antigens was observed in other ATL cells [20].

Discussion

More than 35 years have passed since ATL was found and HTLV-1 was identified as its causative virus several years later. After that, a better molecular understanding of ATL pathology has been advancing. However, at the forefront of clinical practice, many problematic issues, such as a correct diagnosis of smoldering ATL, discrimination from unusual carriers with a percentage of ATL-like cells and promising therapeutic strategies, remain unclear. Recently, understanding of ATL pathology has deepened, but there is no point of contact between clinical and molecular aspects.

The results of the present study revealed that SM was heterogeneous in clonally related SBH features (mainly clone size) and lymphocyte subset profiles. We here designated such cases as monoclonal, oligoclonal and polyclonal smoldering (SM) subtypes. Although there was no difference in clinical manifestations, increase of only





**Fig. 8** Four-color flow cytometry for CD4, CD25, CCR4 and CD26. After CD4 gating, gating-CD4 T-cells were developed into a cytograph (a CCR4 = X-axis, and CD25 = Y-axis), and then P1

gating cells were developed into a cytograph (b CD26 down-regulation positive (blue P3 area meaning loss of CD26 antigen) or negative (red P4 area))

HTLV-1 provirus-carrying cells with a phenotype of  $CD4^+CD25^+CCR4^+CD26^-$  was characteristic, regardless of the stable lymphocyte counts. Moreover, the ratio of CD26/CD25 was defined to be useful as an indicator of the grade of advance into ATL. Such findings were observed partially in unusual carriers with oligoclonal bands. These suggest that the expansion of leukemic clone begins in the unusual carrier stage and reaches large clone detected by SBH analysis in the SM stage. Thus, continuous changes of all ATL-related biomarkers would be explained by growing leukemic clonal cell population [15]. This is easily understood by a diagram shown in Figs. 3 and 4, which was derived from the increase of absolute CD4 T-cells infected by HTLV-1. The SM period seems to oncologically mean one of the turning points for multi-step leukemogenesis of ATL.

Now, it is interesting to develop such a subtype manifestations. Although clinical over-diagnosis cannot be completely neglected, there are in fact such cases with a highly dense smear for Eco-R1 genomic fragments and internal bands for Pst-1 genomic fragments, like the case in Fig. 2. As a possibility, a cluster of

small clones may work co-operatively to develop SM manifestations. Subsequently, this appears to give rise to frequently multiclonal ATL and genomic diversity of leukemic clones [16].

Another interest is the behavior of CCR4, CD7 and CD26. So far, little is known about CD26 associated with ATL pathology. CD26/dipeptide peptidase IV (DPP/IV), which is an antigenic enzyme expressed on the surface of most cell types, suppresses the development of cancer and tumors. CD26 plays an important role in tumor biology, and is useful as a marker for various cancers [17–19]. Now, why would down-regulation of CD26 first occur? The down-regulation preceding the increase in HTLV-1-infected CD4 T-cells may be indispensable to an environment for growing immature ATL cells. On the other hand, down-regulation of CD7, a glycoprotein member of the immunoglobulin (Ig) superfamily, is also one of the most commonly seen antigenic aberrations in T-lymphoproliferative disorders, but there is no specificity for malignant cell types, including a variety of reactive conditions [20, 21]. The changes in expression of CCR4 and CD26 may be the results of transformation.

Furthermore, to reveal other roles of CD26, statistical methodology and a dot plot were used. Consequently, two twin dot-plot graphs of CD25 versus CD26, and HTLV-1-CD4 T-cell number versus the ratio of CD26/CD25 revealed that the ratio of CD26/CD25 is useful as a surrogate marker for the prediction of the provirus clonal status. When the COV of the ratio is 1.04, the diagnostic validity is 87.0% in sensitivity and 83% in specificity. However, the ratio of CD26/CD25 in polyclonal SM was widely distributed, indicating that the polyclonal SM was distinctive from the other two subtypes of oligo- and monoclonal SM. This simple predictive method, alternative to the SBH test which is time- and labor-consuming, may be practically useful for screening in rapid turn-around test or epidemiological mass test.

Finally, using four-color flow cytometry, the usefulness of the CD26 antigen monitor was verified in actual cases. The antigen status was evaluated as the ratio of CD26<sup>(-)</sup> versus CD26<sup>(+)</sup> within a fraction of CD4<sup>(+)</sup>CD25<sup>(+)</sup>CCR4<sup>(+)</sup> cells. The ratio went up with increases in the CD4<sup>(+)</sup>CD25<sup>(+)</sup>CCR4<sup>(+)</sup> cell populations, reflecting occult ATL cells or transforming cells. In the present study, the border line of the ratio between carriers and patients with SM was about 10.0. That is, if the CD26<sup>(-)</sup>:CD26<sup>(+)</sup> ratio is 10 or more, the case is predicted to be smoldering ATL.

Taken together, the present study showed that smoldering ATL was heterogeneous in a clone size and the quality of its constituent cells. This suggests that it is relevant to classify the current smoldering ATL into two subtypes of SM with or without a monoclonal band. Indeterminate HTLV-1 carriers and smoldering ATL can be discriminated according to the patterns of SBH densitometer images and CD26 antigen status. Moreover, CD26 is expected to be used as a novel biomarker for prediction of clonal bands and discrimination of carriers or SM subtypes. CD26 may become one of the central molecules in understanding the early leukemogenic process.

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**Conflict of interest** The authors have no conflict of interest.

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RESEARCH

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# Paradoxical expression of *IL-28B* mRNA in peripheral blood in human T-cell leukemia virus Type-1 mono-infection and co-infection with hepatitis C Virus

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## Abstract

**Background:** Human T-cell leukemia virus type-1 (HTLV-1) carriers co-infected with and hepatitis C virus (HCV) have been known to be at higher risk of their related diseases than mono-infected individuals. The recent studies clarified that IL-28B polymorphism rs8099917 is associated with not only the HCV therapeutic response by IFN, but also innate immunity and antiviral activity. The aim of our research was to clarify study whether IL-28B gene polymorphism (rs8099917) is associated with HTLV-1/HCV co-infection.

**Results:** The genotyping and viral-serological analysis for 340 individuals showed that IL-28B genotype distribution of rs8099917 SNP did not differ significantly by respective viral infection status. However, the IL-28B mRNA expression level was 3.8 fold higher in HTLV-1 mono-infection than HTLV-1/HCV co-infection. The high expression level was associated with TT (OR, 6.25), while the low expression was associated with co-infection of the two viruses (OR, 9.5). However, there was no association between down-regulation and ATL development (OR, 0.8).

**Conclusion:** HTLV-1 mono-infection up-regulates the expression of IL-28B transcripts in genotype-dependent manner, while HTLV-1/HCV co-infection down-regulates regardless of ATL development.

**Keywords:** IL-28B, IL- $\lambda$ 3, HTLV-1, HCV, SNP

## Introduction

A retrovirus, human T-cell leukemia virus type-1 (HTLV-1), and a positive-strand RNA virus, hepatitis C virus (HCV), are completely different in terms of virologic characteristics. Nevertheless, they play a similar role in the pathogenesis of viral-induced malignant neoplasms, such as adult T-cell leukemia (ATL) in HTLV-1-infected individuals, and hepatocellular carcinoma (HCC) and B-cell lymphoma in HCV-infected individuals, during long-term chronic infections.

Furthermore, it is known that co-infection with HCV and HTLV-1 is frequently observed in an area endemic

for HTLV-1. HCV/HTLV-1 co-infected individuals have been reported to be at higher risk for developing HCC than those infected with HCV alone [1-3]. Although the pathologic mechanism of the co-infection remains to be elucidated, it is thought that the impaired immunity due to HTLV-1 infection may contribute to HCV infection and HCV-related disorders, which is suggested by previous reports. Kohno et al. reported that the severe immunodeficiency and anergic state in patients with ATL may be associated with a functional property of leukemic cells originating from regulatory T-cells expressing CD4, CD25, CCR4, GITR and Foxp3 [4]. Kishihara et al. also reported that impairment of the immune response by HTLV-1 could explain the reduced effectiveness of interferon (IFN) treatment in patients co-infected with HTLV-1 and HCV [5].

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