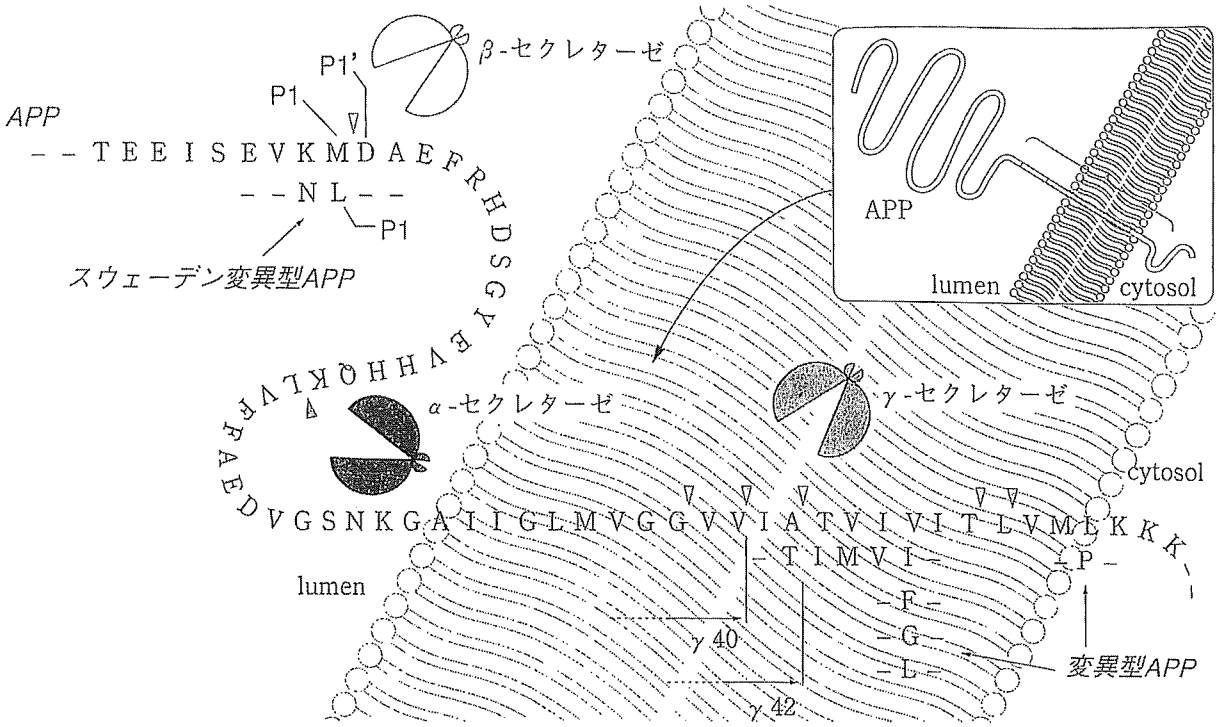


図7　APP切断部位のアミノ酸配列

表2　BACE1のアミノ酸配列 (Vassar R *et al.*, *Science* **286**, 735-741, 1999)

	シグナルペプチド			プロペプチド	
1	MAQALPWLLL	WMGAGVLPAH	GTQHGIRLPL	RSGLGGAPLG	LRLPRETDEE
51	PEEPGRRGSF	VEMVDNLRGK	SGQGYYVEMT	VGSPPQTLNI	LVDTGSSNFA
101	VGAAPHPFLH	RYYQRQLSST	YRDLRKGVYV	PYTQGKWEGE	LGTDLVSIPH
151	GPNVTVRANI	AAITESDKFF	INGSNWEGIL	GLAYAEIARP	DDSLEPFFDS
201	LVKQTHVPNL	FSLQLCGAGF	PLNQSEVLAS	VGGSMIIGGI	DHSLYTGSLW
251	YTPIRREWYY	EVIIVRVEIN	GQDLKMDCKE	YNYDKSIVDS	GTTNLRLPKK
301	VFEAAVKSIK	AASSTEKFPD	GFWLGEQLVC	WQAGTTPWNI	FPVISLYLMG
351	EVTNQSFRIT	ILPQQYLRPV	EDVATSQDDC	YKFAISQSST	GTVMGAVIME
401	GFYVVFDRAR	KRIGFAVSAC	HVHDEFRTAA	VEGPFVTLDM	EDCGYNIPQT
451	DESTLMTIAY	VMAAICALFM	LPLCLMVCQW	RCLRCLRQQH	DDFADDISLL
501	K	膜貫通領域			

どもは、このような研究を長く続けているため、図7をみてすぐわかるのですが、ロイシンの後で切断するのは、アスパギン酸に特徴的です。表2の灰色で示した部分すなわち活性部位にアスパラギン酸があるのは、HIVプロテアーゼと同じです。今、ヨーロッパペプチド学会会長のマルチネス教授が来日していますが、マルチネス教授も「木曽の研究室は山ほど経験があるから、BACE1（β位APP切断酵素）の研究もうまくいっているだろう」といっていました。

β-セクレターゼ阻害剤の開発

さて、β-セクレターゼのエポックメイキングな出来事は1999年の冬に起こりました。その年、β-セクレターゼ活性を有するBACE1がクローニングされ、膜結合型のアスパラギン酸プロテアーゼであることが明らかにされ、阻害剤の研究が一気に加速しました。Aβの蓄積がアルツハイマー病の発症の引き金である

というAβ仮説も、Aβの産生を阻害する薬物を開発できれば真相を明らかにすることができ、アルツハイマー病の治療薬の開発にもつながる可能性があります。

ところで、表2に示すアミノ酸配列から、なぜ、BACE1がアスパラギン酸プロテアーゼであることがすぐにわかるかというと、そこにDTGS（Asp-Thr-Gly-Ser）の配列と、DSGT（Asp-Ser-Gly-Thr）というアミノ酸配列があるためです。Asp-ThrやAsp-Serがこれくらいあればアスパラギン酸プロテアーゼであることはすぐわかります。

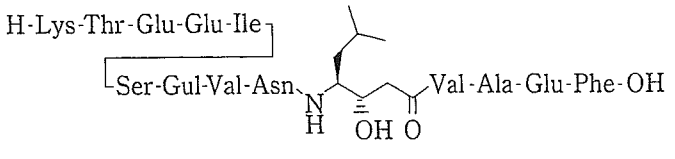
これは重要な活性部位です。われわれの体に発現していますが、特に脳で発現が高くなっています。レニンやペプシンはこんなに複雑ではありません。BACE1は膜に結合もしていますし、システインがあり、糖が付加している難しい酵素です。そのため、阻害剤開発はチャレンジングな問題であることは間違いありません。

アスパラギン酸プロテアーゼにはこれまでの長い歴史があることから、研究者はすぐチャレンジし、オクラホマ・シカゴグループとイーラングループがいっせいに論文を発表しました。世界中が研究をしており、遷移状態をまねる水酸基をもった阻害剤を発表しました（図8）。

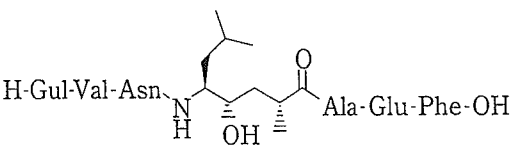
APPのアミノ酸配列に基づいたBACE1阻害剤のデザイン

アスパラギン酸プロテアーゼの反応メカニズムを図9に示します。BACE1の活性部位にアスパラギン酸があり、そこにSer-Glu-Val-Asn-Leu-Asp-Alaというアミノ酸配列をもった基質がくると、Leu-Aspのペプチド結合を切断します。そのときの反応機序は、大学の2年生レベルのやさしい有機化学で、活性部位のアスパラギン酸が攻撃して、電子の流れで、非常にスムーズに反応が進みます。そうすると、P1-P1'のペプチド結合が基質遷移状態を通って切断されます（図9）。日本語で「模倣の」という言葉を、英語では「ミミックまたはミメティック」といいます。アミド結合（CO－NH）のままでは生体内で酵素により切断されてしまうため、C（OH）を挿入しC（OH）－CO－NHとしました（図9）。すなわち、ヒドロキシメチルカルボニル（HMC）イソスター（等価体）です。

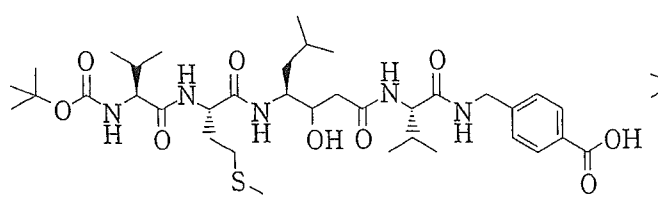
私どもは、レニン阻害剤、HIVプロテアーゼ阻害剤、プラスメプシン阻害剤の研究にこのHMCを使って良好な阻害剤を得ているので、β-セクレターゼをターゲットとして阻害



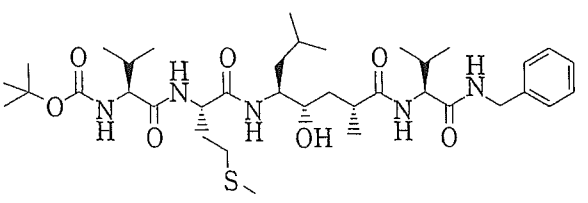
(Sinha S. *et al.*, *Nature* **402**, 537-540, 1999)



(Ghosh A. K. *et al.*, *J. Am. Chem. Soc.* **122**, 3522-3523, 2000)



(Varghese J. *et al.*, WO0077030, 2000)



(Ghosh A. K. *et al.*, *J. Med. Chem.* **44**, 2865-2868, 2001)

図8　β-セクレターゼ阻害剤

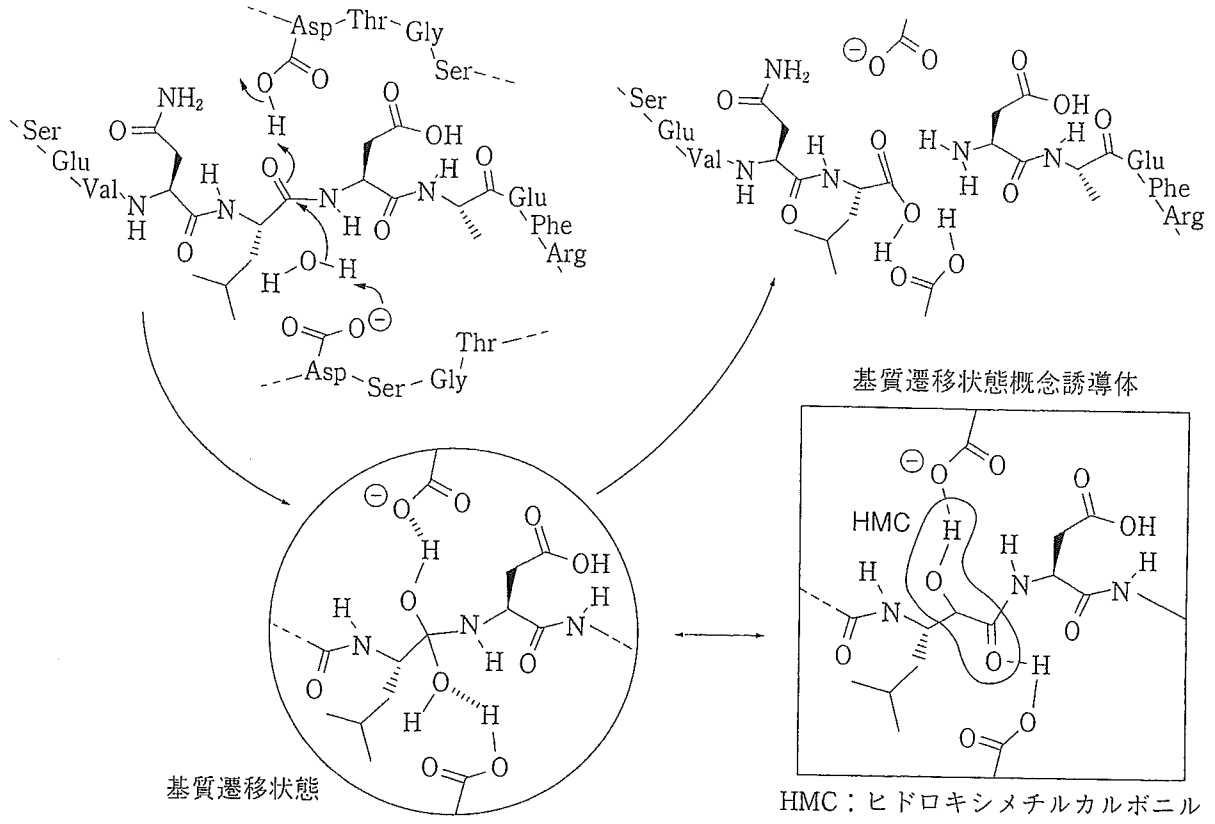


図9　アスパラギン酸プロテアーゼの反応メカニズム

剤の研究を行いました。

HMCのヒドロキシル基（－OH）は、アスパラギン酸のCOO－と水素結合をします。また、そのCOは別のアスパラギン酸のカルボキシル基（－COOH）と水素結合して、酵素反応をこの阻害剤が止めてしまいます。図9左は基質の遷移状態ですから切断されますが、右の遷移状態誘導体は反応が進行しません。非常に簡単な考え方です（図9）。ただし、簡単とはいってもいろいろなやり方があります。人によっては、図10のようなものを使います。梅澤濱夫先生が初めて発見して命名したスタチンは、γ-アミノ酸です。

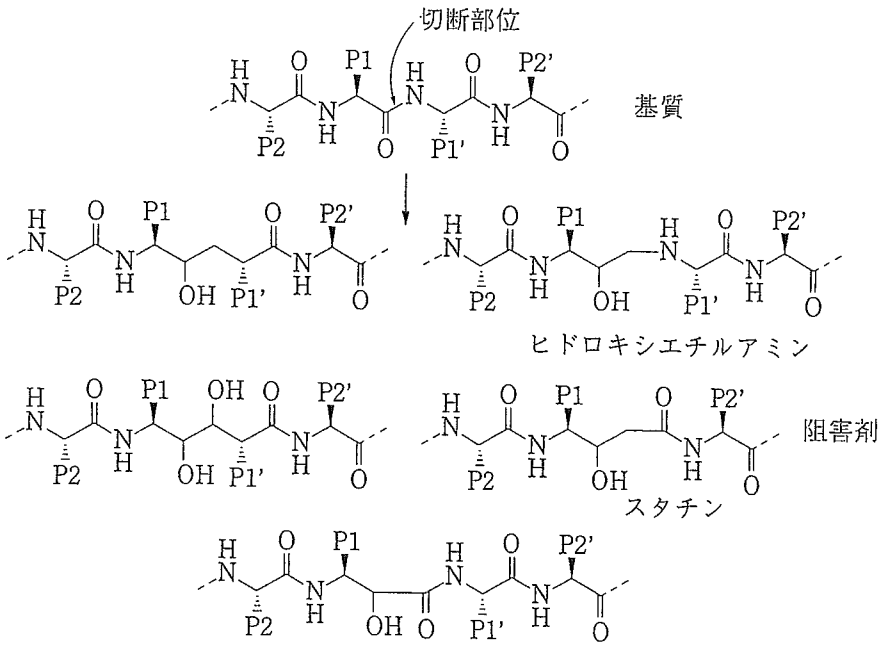


図10　基質遷移状態ミメティック

ちなみに、現在、スタチン系という名前は違う意味に使われています。あまりにも有名な薬のプラバスタチンやシンバスタチンが開発されたためです。「止める」という意味の“スタット”というラテン語に由来して梅澤先生が最初に命名したのが、ペプシンの阻害剤とい

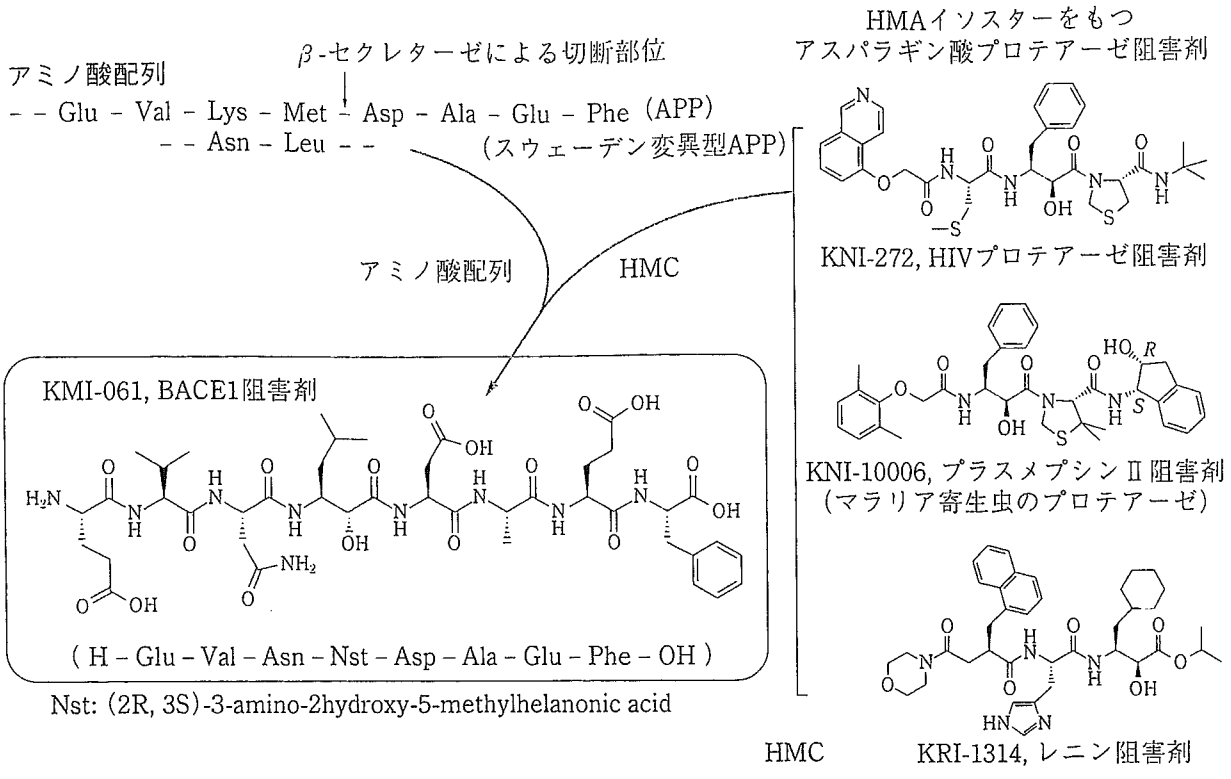


図11　APPのアミノ酸配列に基づいたBACE1阻害剤のデザイン

表3　オクタペプチド型BACE1阻害剤

compound no.	P4 P3 P2 P1 P1' P2' P3' P4'	BACE1阻害(%) (at 2μM)
1 (KMI-061)	H-Glu-Val-Asn-Nst-Asp-Ala-Glu-Phe-OH	< 20
2 (KMI-062)	H-Glu-Val-Asn-Pns-Asp-Ala-Glu-Phe-OH	24
3	H-Glu-Val-Asp-Pns-Asp-Ala-Glu-Phe-OH	25
4	H-Glu-Val-Gln-Pns-Asp-Ala-Glu-Phe-OH	36
5	H-Glu-Val-Glu-Pns-Asp-Ala-Glu-Phe-OH	30
6	H-Glu-Val-Met-Pns-Asp-Ala-Glu-Phe-OH	42
7 (KMI-008)	H-Glu-Val-Leu-Pns-Asp-Ala-Glu-Phe-OH	> 90*
8	H-Glu-Val-Lys-Pns-Asp-Ala-Glu-Phe-OH	< 20
9	H-Glu-Leu-Leu-Pns-Asp-Ala-Glu-Phe-OH	< 20
10	H-Glu-Ile-Leu-Pns-Asp-Ala-Glu-Phe-OH	26
11	H-Glu-Phe-Leu-Pns-Asp-Ala-Glu-Phe-OH	< 20
12	H-Glu-Met-Leu-Pns-Asp-Ala-Glu-Phe-OH	< 20
13	H-Glu-Val-Leu-Pns-Ala-Val-Glu-Phe-OH	30
14	H-Glu-Val-Leu-Pns-Ala-Leu-Glu-Phe-OH	41
15	H-Glu-Val-Leu-Pns-Ala-Phe-Glu-Phe-OH	60
16	H-Glu-Val-Leu-Pns-Ala-Glu-Glu-Phe-OH	78
17	H-Glu-Val-Leu-Pns-Asp-Ala-Gln-Phe-OH	56
18	H-Glu-Val-Leu-Pns-Asp-Ala-Val-Phe-OH	57
19	H-Glu-Val-Leu-Pns-Asp-Ala-Leu-Phe-OH	57
20	H-Glu-Val-Leu-Pns-Asp-Ala-Phe-Phe-OH	67

* (IC_{50} = 413nM)

うことから「ペプスタチン」で、それに含まれていたγ-アミノ酸が「スタチン」と名づけられました。その命名以来、化学の世界では抑制因子を「○○○スタチン」と命名するようになり、たとえば脳内の成長ホルモン放出抑制ホルモンにソマトスタチン、それから開発された薬にサンドスタチンといった命名です。

フェニルノルスタチンを含むBACE阻害剤

APPの家族性アルツハイマー病変異のひとつであるスウェーデン変異型は、β-セクレタ

ーゼに対する親和性が高くAβを産生しやすいことから、私どもはスウェーデン変異型APP配列をもとに、そのP1部位に基質遷移状態誘導体を組み込んだ阻害剤をデザインしました（図11）。残念ながら、KMI-061には活性がほとんど認められませんでした。少し大きめのものにすると、少し活性が上がりました。

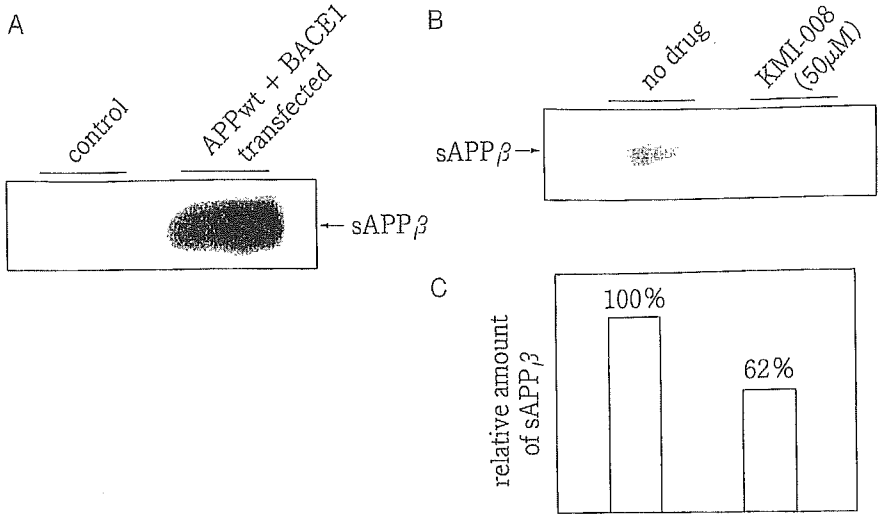


図12　KMI-008のβ-セクレターゼに及ぼす影響　A：APPとBACE1を導入したCOS-7細胞からのsAPPβの分泌（電気泳動のパターン）
B：KMI-008添加によるsAPPβの減少（電気泳動のパターン）。APPとBACE1を導入したCOS-7細胞にKMI-008を加えると（右）、加えない場合（左）に比べてsAPPβの有利が減少していることがわかる。Cの図はこれを定量的に表したもので、加えない場合を100％とするとKMI-008を加えるとsAPPβの遊離が62％に減少している

このような研究は、ポスドクもいますが、大学院生を指導してやっています。大学院生にテーマを与えるとき学生の希望を聞きます。チャレンジングだからうまくいくかどうかわかりませんよといったのですが、院生S君は先の化合物をもとにアミノ酸置換による最適化を行っていったところ、遷移状態誘導体のフェニルノルスタチン（Pns）を含むオクタペプチド型阻害剤KMI化合物を合成しました（表3）。これらのうちKMI-008と名づけた化合物に強い酵素阻害活性が見い出されました。S君には安全なテーマも与えていたのですが、もうこちらにのめりこんでいきました。ポスドクなんかにも教えるぐらいのレベルで、どんどん研究をしてくれました。

細胞ではどうかということを、東京大学の石浦章一先生にCOS-7細胞を用いてAPPβが切れたものを解析してもらいました（図12）。その結果、KMI-008を添加すると、弱いものの確かに阻害しました。細胞でもβ-セクレターゼ阻害活性をもっていたわけです。

低分子型BACE阻害剤

KMI-008は、アミノ酸は8個ですから、これでは薬にはなりません。小さくする必要があります。そこで、活性発現に必要な最小構造を求めるために、KMI-008からアミノ酸を切除して低分子化を行いました（表4）。

KMI-008のような化合物ができると、みんなが研究をやりたがったため、あっという間に研究が進み、P4からP1'までのリガンドを有するペンタペプチド誘導体でも、確かな活性を有していることがわかりました。

この結果は、同じアスパラギン酸プロテアーゼであるレニンの阻害剤の構造活性相関とも共通する点があり、非常に興味深くなっています。

ただし、このペプチドだけでは生体での安定性が悪いことから、さらに構造の最適化を進めていきました。そして、グルタミン酸（Glu）のかわりにオキザリルで置換したジアミノプロピオン酸を導入しました（図13）。C末端もカルボン酸を普通のアミノ酸にしたのでは活性が上がらないため、アミノ安息香酸にしたところ、非常に強い、チャンピオンデータレベルまで活性が上がりました。3.4nMという非常に強い活性があります。

細かい話をすると、オクラホマグループの化合物OM99-2と私どもの化合物では、C末端がだいぶ違います(図14)。ですから、選択性などがかなり違っています。石浦先生にHEK293細胞を使って、この化合物(KMI-358およびKMI-370)のβ-セクレターゼ活性を調べていただいたところ、濃度依存的にβ-セクレターゼを阻害していました(図15)。

一方、KMI-358とKMI-370のオキザリル基は転移しやすいため、カルボン酸とよく似たものを導入しました。カルボン酸イソスターとして、武田薬品の血圧降下剤アンジオテンシン受容体の遮断薬で仲健彦先生が使われ、世界中の人が使っているテトラゾールを導入してみました。

その結果、得られたKMI-420は予想どおり、非常に活性がよくなっています。しかも、化学的な安定性も上がって、非常によいことがわかりました。

さらに、カルボン酸2つとテトラゾールをもつKMI-429も非常に優れています(図16)。先日、京都でアルツハイマー病協会の国際会議があり、米国の研究者や会議の副委員長などたくさんの人がKMI-429に注目され、米国では2次情報にかなり詳しくでるという話です。

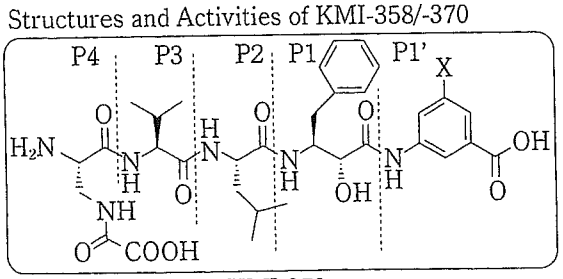
日本生化学会では石浦章一先生が発表されたので、そちらからの問い合わせも多くなっています。現在、ライセンスして欲しいという要請が結構きています。

相互作用をみると、このテトラゾールがうまくいっている(図17)ことから、この考え方は間違っていないことがわかりました。これ

表4　最小活性構造の探索

compound no.	P4 P3 P2 P1 P1' P2' P3' P4'	BACE1阻害(%) (at 2μM)
7 (KMI-008)	H-Glu-Val-Leu-Pns-Asp-Ala-Glu-Phe-OH	> 90
32	H-Val-Leu-Pns-Asp-Ala-Glu-Phe-OH	< 20
33	H-Leu-Pns-Asp-Ala-Glu-Phe-OH	< 20
34	H-Pns-Asp-Ala-Glu-Phe-OH	< 20
35	H-Glu-Val-Leu-Pns-Asp-Ala-Glu-OH	60
36	H-Glu-Val-Leu-Pns-Asp-Ala-OH	46
37	H-Glu-Val-Leu-Pns-Asp-OH	61
38	H-Glu-Val-Leu-Pns-OH	34
39	H-Gly-Val-Leu-Pns-Asp-Ala-Glu-Phe-OH	< 20
40	H-Glu-Gly-Leu-Pns-Asp-Ala-Glu-Phe-OH	< 20
41	H-Glu-Val-Gly-Pns-Asp-Ala-Glu-Phe-OH	< 20
42	H-Glu-Val-Leu-Pns-Gly-Ala-Glu-Phe-OH	54
43	H-Glu-Val-Leu-Pns-Asp-Gly-Glu-Phe-OH	73
44	H-Glu-Val-Leu-Pns-Asp-Ala-Gly-Phe-OH	56
45	H-Glu-Val-Leu-Pns-Asp-Ala-Glu-Gly-OH	65

Structures and Activities of KMI-358/-370



	X	BACE1 inhibition IC_{50}(nM)
KMI-358	–H	16
KMI-370	–COOH	3.4

Synthetic Route to KMI-370

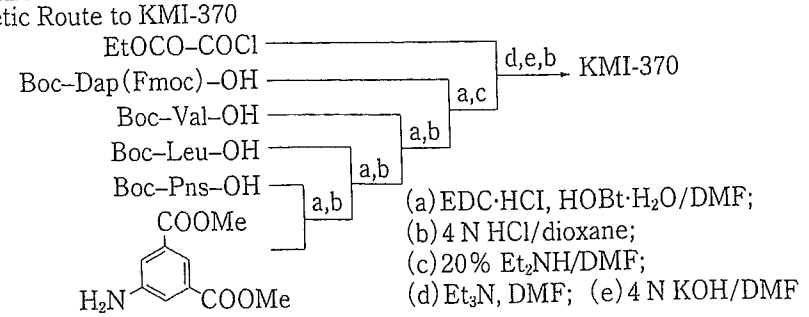


図13　小分子型BACE1阻害剤、KMI-358/-370

は薬としては途中段階であると思いますが、可能性はあるという状況です。

γ-セクレターゼ阻害剤の開発

γ-セクレターゼについては簡単に触れます。

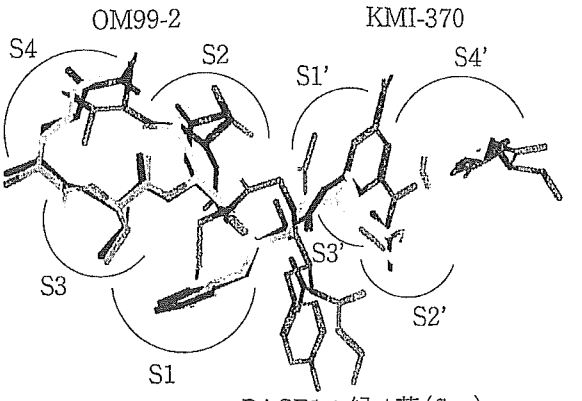
γ-セクレターゼにはアスパラギン酸プロテアーゼ活性も認められ、その阻害剤も活発に開発されていますが、タンパク質複合体と考えられていて、いまだ正体は不明です。また、γ-セクレターゼは、発生・分化に深く関与している1回膜貫通型タンパク質のいくつかを基質にすることが知られています。実際、γ-セクレターゼの主なタンパク質部分と考えられているプレセニリンのノックアウトマウスは体節異常をきたし、胎生致死です。以上のことから、γ-セクレターゼを安易に阻害することは好ましくないとの考え方もあります。

APPには、γ-セクレターゼによる切断部位がたくさん存在しています。そのため、β-セクレターゼ阻害研究に比べたら論理性が少し難しくなります。

ただし、γ-セクレターゼ阻害剤として非ステロイド抗炎症薬（NSAID）ようなものもあります。活性部位ではなく、ほかの領域に結合してγ-セクレターゼ阻害を示す可能性があることから、このような化合物の研究も進める必要があります。γ-セクレターゼ阻害剤の研究も、結合部位を見つけるためにますますさかんになっています。

今後の課題と展望

基質遷移状態概念に基づいてβ-セクレターゼ阻害剤を設計し、強い活性をもつ化合物を見い出しました。しかし、β-セクレターゼの作用部位を考えると、



BACE1：緑/茶(flap)
OM99-2：黄、KMI-370：青

図14　OM99-2 vs KMI-370（口絵カラー参照）

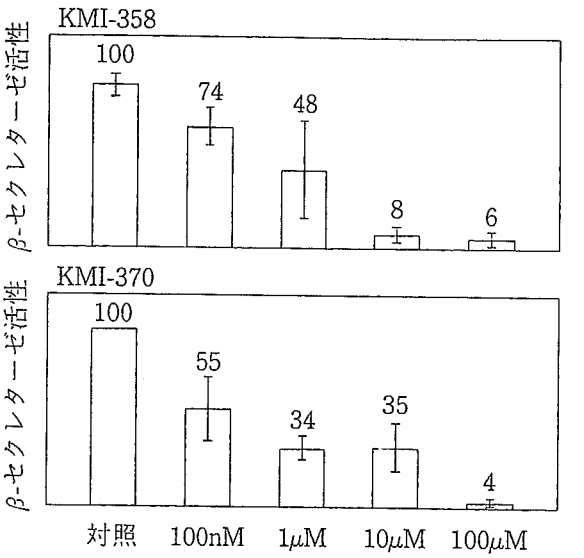
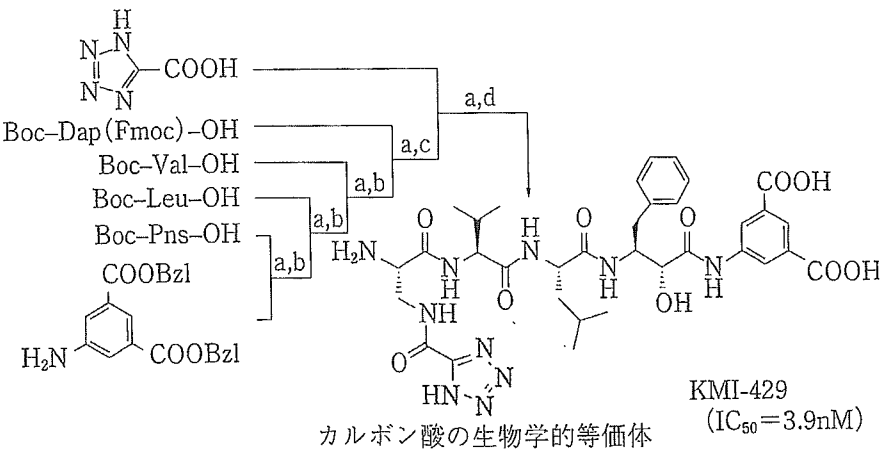


図15　β-セクレターゼ活性に及ぼすKMI-358/-370の効果(in BACE1-HEK293 cells, n＝3)



カルボン酸の生物学的等価体

Abbrev.: Dap; L-α,β-diaminopropionic acid,
Reagents: (a) EDC·HCl, HOBt/DMF; (b) 4N HCl/dioxane;
(c) 20% Et_2NH/DMF; (d) TMS-Br, thioanisole, *m*-cresol/TFA

図16　KMI-429の合成

薬剤は血液脳関門を通過する必要があり、分子サイズや親和性疎水性バランスなど、考慮すべき点が多くなっています。また。従来のアスパラギン酸プロテアーゼと基質特異性が異なり、阻害剤設計も新しいアイディアが必要になると思われます。

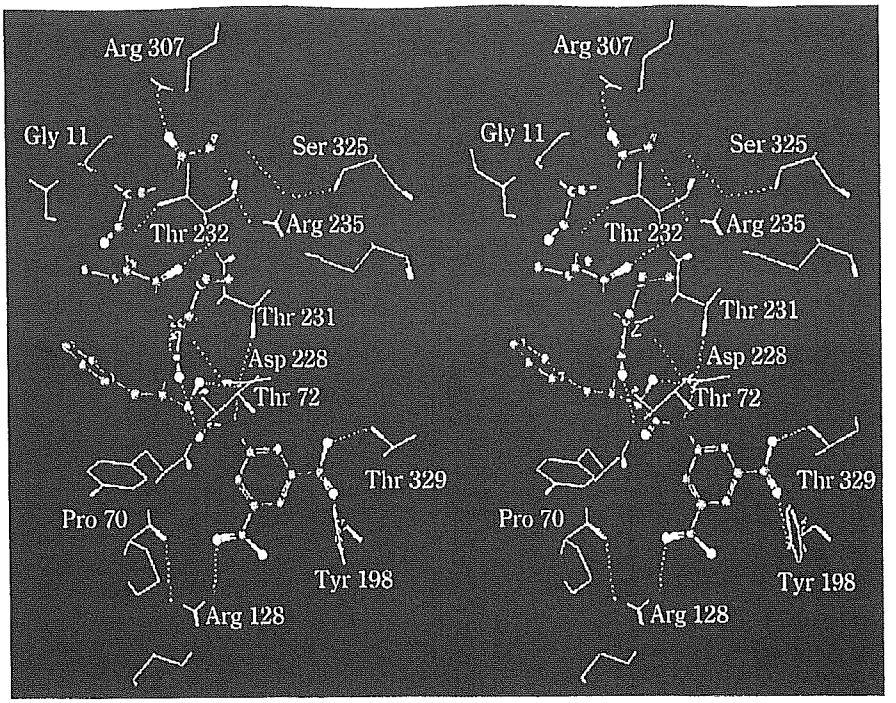


図17　BACE1と結合したKMI-429の分子モデル（ステレオ対）（口絵カラー参照）

HIVプロテアーゼ阻害のときもそうでしたが、1991年に、読売新聞の科学部のデスクがわざわざ京都までこられて、「非常によくわかりました。希望をもたせることは非常に大事ですね」といわれました。そのころ、ほかの先生方が「先生、カウンセラーになれますよ」といっていました。いつも「希望をもってください」といっていたためです。論理性がある薬は、開発に時間がかかるかもしれませんが、希望はあると思いますので、ぜひ、希望をもっていただきたいと思います。阻害剤の開発は、アルツハイマー病発症機構の解明や治療に役立つ可能性があり、今後の発展に期待したいと思います。

石浦先生、理研の橋本先生、西道先生をはじめたくさんの研究者に感謝します。私どもの研究は、文部科学省学術フロンティア推進事業、文部科学省21世紀COEの支援をいただいています。この先端脳では井原康夫先生にお世話になりました。この場を借りてお礼申し上げます。

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AIRWAY BIOLOGY

Bronchoalveolar lymphocytosis correlates with human T lymphotropic virus type I (HTLV-I) proviral DNA load in HTLV-I carriers

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Background: A study was undertaken to investigate the pathogenesis of pulmonary involvement in human T lymphotropic virus type I (HTLV-I) carriers.

Methods: The bronchoalveolar lavage (BAL) cell profile of 30 HTLV-I carriers (15 asymptomatic HTLV-I carriers (AHCs) and 15 symptomatic HTLV-I carriers (SHCs)) with chronic inflammatory diseases of respiratory tract and eight patients with HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) was investigated. The HTLV-I proviral deoxyribonucleic acid (DNA) load in peripheral blood mononuclear cells (PBMCs) and BAL fluid from HTLV-I carriers was estimated using the quantitative polymerase chain reaction method and the correlation between the lymphocyte number in BAL fluid and the HTLV-I proviral DNA load in PBMCs and BAL fluid was examined.

Results: The percentage of lymphocytes in BAL fluid was increased (>18%) in 11 of 30 HTLV-I carriers although there was no significant difference compared with control subjects. In HTLV-I carriers the lymphocyte number in BAL fluid correlated well with the copy number of HTLV-I proviral DNA in PBMCs. In addition, the copy number of HTLV-I proviral DNA in BAL fluid correlated well with the number of lymphocytes (both CD4+ and CD8+ cells) in BAL fluid.

Conclusions: These findings suggest that pulmonary lymphocytosis can occur in a subset of HTLV-I carriers without HAM/TSP and that the increased HTLV-I proviral DNA load may be implicated in the pathogenesis of pulmonary involvement in HTLV-I carriers.

Human T lymphotropic virus type I (HTLV-I) is a type C retrovirus that is aetiologically associated with adult T cell leukaemia[1 2] and with HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP).[3 4] In addition to these diseases, a number of inflammatory disorders have also been described in association with HTLV-I including HTLV-I uveitis,[5] arthropathy,[6] and Sjögren's syndrome.[7] Pulmonary involvement is also associated with HTLV-I—for example, in patients with HAM/TSP and HTLV-I uveitis pulmonary involvement may be characterised by bronchoalveolar lymphocytosis.[8–11] Furthermore, a few preliminary studies have shown that similar pulmonary involvement is observed in HTLV-I carriers who have not developed HAM/TSP or HTLV-I uveitis.[10 12]

Many kinds of immunological abnormalities and an increased HTLV-I proviral deoxyribonucleic acid (DNA) load in peripheral blood, cerebrospinal fluid, and bronchoalveolar lavage (BAL) fluid from patients with HAM/TSP[13 14] and HTLV-I uveitis[11] have been reported, suggesting that immunological mechanisms related to an increased amount of HTLV-I proviral DNA may be implicated in the pathogenesis of these diseases. However, despite advances in elucidating the pathophysiology of these diseases, much of the information on the pathogenesis is confined to HAM/TSP and HTLV-I uveitis. There is little information available regarding pulmonary involvement and pathophysiology in HTLV-I carriers who have not developed HAM/TSP or HTLV-I uveitis.

To examine the incidence and pathogenesis of pulmonary lymphocytosis in HTLV-I carriers, we have analysed BAL cell profiles in HTLV-I carriers including asymptomatic HTLV-I carriers (AHCs). We also estimated the HTLV-I proviral DNA load in peripheral blood mononuclear cells (PBMCs) and BAL cells from HTLV-I carriers by the quantitative polymerase chain reaction (PCR) method and examined the correlation between the HTLV-I proviral DNA load and pulmonary lymphocytosis.

METHODS

This study was reviewed and approved by the Kagoshima University Faculty of Medicine Committee on Human Research.

Study subjects

The study subjects consisted of 30 HTLV-I carriers and eight patients with HAM/TSP consecutively presenting to our department between 1989 and 2000. The 30 HTLV-I carriers consisted of 15 AHCs (three men and 12 women) and 15 symptomatic HTLV-I carriers (SHCs; five men and 10 women) as shown in table 1. There were no significant differences in age between each of the groups and the control subjects. All subjects were seronegative for human immunodeficiency virus (HIV) 1.

To assess the cellular characteristics of BAL fluid in AHCs, the serum anti-HTLV-I antibody was checked in individuals consulting our department for an annual chest radiograph. The anti-HTLV-I antibody was measured by the gelatin particle agglutination method (Fujirebio, Tokyo, Japan). After obtaining informed consent, further examinations including fibreoptic bronchoscopy were performed on HTLV-I seropositive individuals along with careful history taking including occupational history. Individuals who

Abbreviations: AHC, asymptomatic HTLV-I carrier; BAL, bronchoalveolar lavage; HAM/TSP, HTLV-I associated myelopathy/tropical spastic paraparesis; HTLV-I, human T lymphotropic virus type I; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; SHC, symptomatic HTLV-I carrier

Table 1 Clinical background and bronchoalveolar lavage (BAL) findings of HTLV-I carriers and patients with HAM/TSP

Patient no. and clinical diagnosis/symptoms	Age/ sex	Peripheral blood: WBC (/mm³)	HTLV-I Ab‡ (×)	Cell count (×10⁵/ml)	Bronchoalveolar lavage fluid: AM (%)	Ly (%)	Neu (%)	Eo (%)	CD4 (%)	CD8 (%)	CD4/CD8
1 AHC	64/F	4300	2048	1.4	94.3	5.0	0.8	0.0	ND	ND	ND
2 AHC†	77/M	7500	2048	1.0	96.4	3.2	0.4	0.0	52.0	32.9	1.6
3 AHC	72/F	5800	256	2.0	90.2	8.3	1.3	0.2	47.1	27.3	1.7
4 AHC	48/F	2600	512	0.4	86.9	12.3	0.6	0.1	48.9	29.2	1.7
5 AHC	57/F	6500	1024	0.7	74.9	24.6	0.5	0.0	53.5	25.0	2.1
6 AHC	78/F	6300	8192	1.9	88.2	10.5	0.8	0.0	53.0	28.3	1.9
7 AHC	58/F	3600	8192	0.9	84.4	14.3	1.2	0.0	ND	ND	ND
8 AHC†	64/M	4300	512	0.7	96.2	3.3	0.5	0.0	ND	ND	ND
9 AHC	71/M	5400	4096	2.8	70.5	28.6	0.9	0.1	75.6	23.2	3.3
10 AHC	56/F	7000	128	1.0	80.5	18.7	0.6	0.1	44.5	39.5	1.1
11 AHC	64/F	4000	128	1.3	89.2	9.7	0.8	0.3	58.8	22.8	2.6
12 AHC	55/F	3700	2048	0.6	95.3	4.0	0.3	0.5	ND	ND	ND
13 AHC	77/F	4300	16384	1.5	76.1	23.4	0.4	0.1	75.3	13.4	5.6
14 AHC	71/F	5300	4096	0.6	84.3	13.5	1.6	0.5	64.6	26.9	2.4
15 AHC	67/F	4700	1024	0.8	91.5	5.5	1.2	0.0	42.8	17.1	2.5
16 Chronic cough	76/F	5100	256	1.0	93.6	5.7	0.7	0.0	37.1	34.8	1.1
17 Chronic cough	67/F	3900	1024	0.9	68.5	30.1	1.4	0.0	71.7	25.9	2.8
18 Chronic cough*	82/F	7000	128	1.7	95.2	4.7	0.2	0.0	ND	ND	ND
19 Middle lobe syndrome	59/F	5200	4096	0.7	61.0	34.6	3.4	0.0	55.8	21.1	2.6
20 SBS	71/F	3400	4096	0.5	92.0	7.1	0.8	0.2	48.4	31.7	1.5
21 Bronchiectasis	63/F	2800	2048	0.6	94.9	4.9	0.1	0.0	40.4	42.1	1.0
22 Inactive Tbc	57/M	3900	1024	1.3	72.7	27.3	0.0	0.0	57.1	40.5	1.4
23 Inactive Tbc†	73/M	7000	2048	3.0	94.9	4.7	0.4	0.0	60.0	30.8	2.0
24 Chronic bronchitis	47/M	4700	256	0.6	91.6	8.1	0.2	0.0	ND	ND	ND
25 Chronic bronchitis*	57/M	5200	256	1.8	87.0	11.4	1.4	0.2	ND	ND	ND
26 Bronchiectasis	72/F	5000	512	1.9	97.4	2.6	0.0	0.0	ND	ND	ND
27 Bronchiectasis	68/F	6000	128	1.5	55.1	29.6	13.1	1.2	57.5	22.9	2.5
28 SBS	46/M	6900	2048	5.8	42.5	20.3	25.0	0.6	41.1	49.7	0.8
29 Middle lobe syndrome	66/F	6300	512	2.5	44.4	55.3	0.1	0.2	61.9	29.6	2.1
30 SBS	18/F	7900	4096	2.7	47.6	45.5	7.1	0.3	41.6	55.2	0.8
31 HAM/TSP	33/F	4100	2048	2.0	42.7	56.8	0.2	0.2	43.1	51.5	0.8
32 HAM/TSP	50/F	4900	2048	2.3	65.6	21.0	13.4	0.0	46.4	49.2	0.9
33 HAM/TSP	54/F	2800	512	1.4	63.0	36.0	1.0	0.0	69.1	28.5	2.4
34 HAM/TSP	60/M	5100	512	2.4	73.4	24.5	2.1	0.0	47.1	48.2	1.0
35 HAM/TSP	65/F	5200	2048	1.7	40.8	58.9	0.3	0.0	33.1	61.0	0.5
36 HAM/TSP	59/F	4900	32768	4.0	27.0	71.9	1.1	0.0	76.2	20.4	3.7
37 HAM/TSP	34/M	5600	8192	1.6	73.2	25.7	1.2	0.0	50.2	42.1	1.2
38 HAM/TSP	50/M	6100	8192	3.9	31.2	68.3	0.0	0.5	52.0	46.9	1.1

WBC, white blood cell; M, male; F, female; HTLV-I, human T lymphotropic virus type I; AHC, asymptomatic HTLV-I carrier; HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis; SBS, sinobronchial syndrome; Tbc, tuberculosis; AM, alveolar macrophage; Ly, lymphocyte; Neu, neutrophil; Eo, eosinophil; ND, not determined.
*Current smoker.
†Recent/ex-smoker.
‡HTLV-I antibody (Ab) was measured by the gelatin particle agglutination method.

worked in environments known to cause allergic lung diseases were excluded. After the diagnostic procedure, 15 AHCs were recruited to the study (table 1); the chest radiographic findings were normal in 13 individuals and two had minimal inactive tuberous lesions.

The 15 SHCs (subjects 16–30) were recruited from the outpatient clinic of our department for chronic inflammatory diseases of the respiratory tract (three sinobronchial syndrome, three bronchiectasis, two middle lobe syndrome, and two chronic bronchitis); two had an inactive tuberculous lesion and three (subjects 16–18) complained of a slight cough for 1–3 months during the study. Eight patients with HAM/TSP (three men and five women) had been diagnosed according to the criteria proposed by Osame *et al.*[15] Three HTLV-I carriers (subjects 9, 18 and 25) were current smokers, three HTLV-I carriers (subjects 2, 8 and 23) were ex-smokers with intervals ranging from 3 months to 5 years since smoking cessation, and the others had never smoked.

White blood cell counts in peripheral blood were within the normal range in all subjects. The serum anti-HTLV-I antibody titre ranged from 128 to 16384× in HTLV-I carriers and from 512 to 32768× in patients with HAM/TSP. The median (range) % vital capacity of AHCs, SHCs, and patients with HAM/TSP was 104.9% (77.2–131.8), 85.8% (75.9–131.3), and 96.7% (76.7–108.0), respectively, and the % forced expiratory volume in 1 second in the three groups was 82.2% (79.3–91.7), 70.7% (51.6–86.2), and 79.7% (70.0–88.3%), respectively. There were no significant differences in pulmonary function between the three groups.

Nine healthy individuals (three men and six women) of median age 53 years, all non-smokers, who were seronegative for HTLV-I acted as controls. They included four healthy volunteers and five healthy individuals undergoing an annual chest radiographic examination. These latter five healthy individuals were finally diagnosed as having a small solitary lung nodule without signs of pulmonary disease (n = 3) or minimal inactive tuberculous lesions (n = 2). BAL was performed to diagnose the small lung nodule and the cellular characteristics of the BAL fluid from these subjects served as

Table 2 Oligonucleotides for PCR detection of HTLV-I proviral DNA

Function	Nucleotide sequence (5' to 3')	Position*
Primer	GGC TCC GTT GTC TGC ATG TA	7765–7784
Primer	AAT CAT AGG CGT GCC ATC GG	8091–8072
Probe	CCT AAT AAT TCT ACC CGA AGA CTG TTT GCC	7932–7961

*GenBank Accession No. J02029.
PCR, polymerase chain reaction; HTLV-I, human T lymphotropic virus type I.

control values for this study. The chest radiographic findings of the five healthy volunteers were normal.

Bronchoalveolar lavage

Informed consent was obtained from all individuals before they underwent BAL which was performed before interventions including corticosteroid administration. Under local anaesthesia with 2% lidocaine, a fibreoptic bronchoscope was placed in the subsegment of the right middle lobe or lingua and 160 ml sterile saline was infused in four aliquots through the bronchoscope and aspirated by gentle hand suction. The lavage fluid obtained was passed through two sheets of sterile gauze and a 10 ml aliquot was centrifuged at 400*g* for 10 minutes, stained with Wright-Giemsa stain, and the cell differentials were determined (at least 500 cells were counted). The lavage fluid was then washed twice and the total number of cells counted.

An aliquot of BAL cells was used for identification of T lymphocyte subsets. The cells were washed twice with phosphate buffered saline and incubated with an optimal concentration of fluorescence conjugated monoclonal antibodies (OKT4 (CD4), OKT8 (CD8); Ortho Diagnostics, Raritan, NJ, USA). The cells were then analysed for surface fluorescence using flow cytometry (FCMID, Nihon Bunko, Tokyo, Japan).

Isolation of peripheral blood mononuclear cells (PBMCs)

The PBMCs were isolated from 30 ml heparinised peripheral blood by Ficoll-Hypaque density gradient centrifugation (Pharmatica, Uppsala, Sweden). Blood samples were obtained before performing BAL in all subjects. These samples were stored in liquid nitrogen until use.

Quantitative PCR of PBMCs and BAL cells

Quantitative PCR assay was performed as previously described.[16] The amount of HTLV-I proviral DNA was calculated using the following formula: copy number of HTLV-I (pX) per 10^4 PBMCs and per 10^4 BAL cells = [(copy number of pX)/(copy number of β-actin/2)] × 10^4

Detection of HTLV-I proviral DNA from BAL cells by PCR

To examine the presence of HTLV-I proviral DNA in BAL cells, isolated BAL cells from 10 initial AHCs (nos 1–9 and 11) were analysed. As negative and positive controls, BAL cells and HTLV-I infected TCL-Kan cells[17] from HTLV-I seronegative controls were also assayed. 1 µg of DNA extracted from BAL cells was used for the PCR. The amplification reaction of PCR was performed for 35 cycles and consisted of denaturation at 94°C for 1 minute, annealing at 65°C for 2 minutes, and primer extension at 72°C for 3 minutes. 10 µl of the amplified products was blotted onto a nylon membrane and hybridised with a biotin labelled probe for HTLV-I pX. After incubation with streptavidin-alkaline phosphate conjugate, disodium 3-(4-methoxyspirol [1,2-dioxetane-3-2′-tricyclo-[3.3.1.1.3.7] decan]-4-yl)phenyl phosphate (AMPPD)[18] (Southern Light Kit, Troix) was added to the blot to a final concentration of 0.25 mM and the immersed blot was then slowly agitated for 5 minutes. Finally, the chemiluminescent signal was detected by exposing the radiographic film.

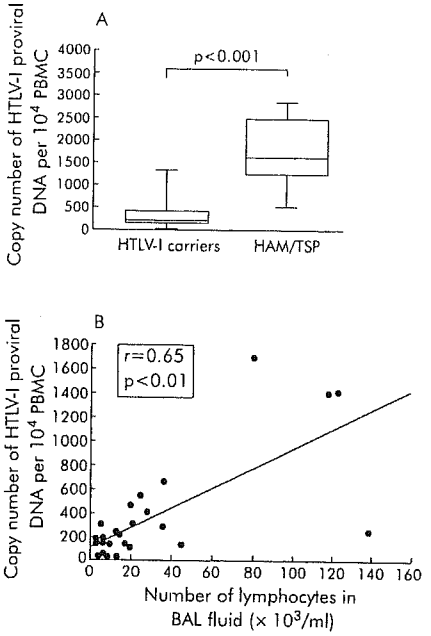

Figure 1 (A) Copy number of HTLV-I proviral DNA per 10^4 PBMCs in HTLV-I carriers and patients with HAM/TSP. The whisker box plots represent the 25–75th percentile of results inside the box, the median is shown by the horizontal bar across the box, and whiskers on the box represent the 10–90th percentiles. (B) Correlation between the number of lymphocytes in bronchoalveolar lavage (BAL) fluid and the copy number of HTLV-I proviral DNA per 10^4 PBMCs in HTLV-I carriers. HTLV-I, human T lymphotropic virus type I; PBMCs, peripheral blood mononuclear cells; HAM/TSP = HTLV-I associated myelopathy/tropical spastic paraparesis.

Table 3 Bronchoalveolar lavage findings in HTLV-I carriers and HAM/TSP patients

Subjects	Cell count (×10^5/ml)	AM (%)	Ly (%)	Neu (%)	Eo (%)	CD4+ cells (%)	CD8+ cells (%)	CD4/CD8
HTLV-I carriers	1.2**	87.6	11.0	0.8	0.0	53.3	28.8	1.9
N=30 (22)†	(0.4–5.8)	(42.5–97.4)	(2.6–55.3)	(0.0–25.0)	(0.0–1.2)	(37.1–75.6)	(13.4–55.2)	(0.8–5.6)
AHCs	1.0*	88.2	10.5	0.8	0.1	53.0	26.9	2.1*
N=15 (11)†	(0.4–2.8)	(70.5–96.4)	(3.2–28.6)	(0.3–1.6)	(0.0–0.5)	(42.8–75.6)	(13.4–39.5)	(1.1–5.6)
SHCs	1.5**	87.0	11.4	0.7	0.0	55.8	31.7	1.5
N=15 (11)†	(0.5–5.8)	(42.5–97.4)	(2.6–55.3)	(0.0–25.0)	(0.0–1.2)	(37.1–71.7)	(21.1–55.2)	(0.8–2.8)
HAM/TSP	2.2**	52.9**	46.4**	1.1	0.0	48.7	47.6*	1.0
N=8	(1.4–4.0)	(27.0–73.4)	(21.0–71.9)	(0.0–13.4)	(0.0–0.5)	(33.1–76.2)	(20.4–61.0)	(0.5–3.7)
Controls	0.6	91.2	8.4	0.6	0.1	48.7	31.4	1.5
N=9	(0.3–1.0)	(87.9–95.8)	(3.8–10.5)	(0.2–1.3)	(0.0–0.4)	(33.4–59.5)	(22.7–45.1)	(0.9–2.1)

Data are shown as median (range).
N, number of subjects; AM, alveolar macrophage; Ly, lymphocyte; Neu, neutrophil; Eo, eosinophil; HTLV-I, human T lymphotropic virus type I; AHCs, asymptomatic HTLV-I carriers; SHCs, symptomatic HTLV-I carriers with chronic inflammatory diseases of respiratory tract; HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis.
*p<0.05, **p<0.01 compared with control subjects.
†The cell differential in BAL fluid was determined in 30 HTLV-I carriers (15 AHCs and 15 SHCs) and T lymphocyte subsets were determined in 22 HTLV-I carriers (11 AHCs and 11 SHCs).

Table 4 Quantification of HTLV-I proviral DNA in PBMCs and BAL fluid from HTLV-I carriers and HAM/TSP patients

Subjects	PBMCs			BAL fluid		
	N	Median	Range	N	Median	Range
HTLV-I carriers	26	199	ND–1704	14	126	22–1268
AHCs	11	138	ND–1704	3	75	70–132
SHCs	15	232	30–1390	11	141	22–1268
HAM/TSP	8	1611*	261–2857	8	601**	294–3495

HTLV-I copy number per 10^4 PBMCs and per 10^4 BAL cells are presented.
HTLV-I, human T lymphotropic virus type I; AHCs, asymptomatic HTLV-I carriers; SHCs, symptomatic HTLV-I carriers with chronic inflammatory diseases of respiratory tract; HAM/TSP, HTLV-I associated myelopathy/tropical spastic paraparesis; PBMCs, peripheral blood mononuclear cells; BAL, bronchoalveolar lavage; N, number of subjects; ND, not detected.
*$p<0.01$ compared with HTLV-I carriers, AHCs and SHCs; **$p<0.01$ compared with HTLV-I carriers and SHCs.

The location and sequences of the primers and probe are summarised in table 2.

Statistical analysis

All values are shown as median (range). Statistical analysis was performed using the Mann-Whitney U test and Spearman rank correlation. p values of <0.05 were considered significant.

RESULTS

Cellular characteristics and T lymphocyte subset of BAL fluid

The recovery ratios of BAL fluid in HTLV-I carriers, AHCs, SHCs, and patients with HAM/TSP did not differ significantly from those of control subjects (data not shown). As shown in table 3, the BAL fluid cell count was increased in HTLV-I carriers, AHCs, SHCs, and patients with HAM/TSP compared with control subjects. The differential cell count showed an increased percentage of lymphocytes and a decreased percentage of macrophages in BAL fluid from patients with HAM/TSP compared with control subjects. The percentage of lymphocytes in BAL fluid was increased (>18%) in four AHCs and seven SHCs but there was no significant difference from that in control subjects.

Analysis of T lymphocyte subsets in BAL fluid showed an increased percentage of CD8+ cells in patients with HAM/TSP and an increased ratio of CD4/CD8 in AHCs.

HTLV-I proviral DNA in PBMCs and correlation with lymphocytes in BAL fluid

The copy number of HTLV-I proviral DNA in PBMCs was determined in 26 HTLV-I carriers (11 AHCs and 15 SHCs) and eight patients with HAM/TSP. As shown in table 4 and fig 1A, the copy number of HTLV-I proviral DNA per 10^4 PBMCs was significantly increased in patients with HAM/TSP compared with those of HTLV-I carriers ($p<0.001$), AHCs ($p<0.01$), and SHCs ($p<0.01$). There was no significant difference in the copy number of HTLV-I proviral DNA of PBMCs between AHCs and SHCs.

The relationship between the HTLV-I proviral load in PBMCs and the lymphocyte number in BAL fluid was examined in 26 HTLV-I carriers. As shown in fig 1B, the number of lymphocytes in BAL fluid correlated well with the copy number of HTLV-I proviral DNA in PBMCs of HTLV-I carriers ($r = 0.65$, $p<0.05$).

HTLV-I proviral DNA in BAL fluid and correlation with lymphocytes and T lymphocyte subsets in BAL fluid

HTLV-I proviral DNA was detected by PCR in BAL cells from all 10 AHCs examined and was not detected in BAL cells from HTLV-I seronegative controls.

The copy number of HTLV-I proviral DNA in BAL fluid was determined in 14 HTLV-I carriers (three AHCs and 11 SHCs) and in eight patients with HAM/TSP. As shown in table 4, the copy number of HTLV-I proviral DNA per 10^4 BAL cells was significantly increased in patients with HAM/TSP compared with those of HTLV-I carriers ($p<0.001$) and SHCs ($p<0.01$).

The correlation between HTLV-I proviral DNA load, lymphocyte number, and T cell subsets in BAL fluid was examined in 14 HTLV-I carriers and 10 HTLV-I carriers. The copy number of HTLV-I proviral DNA in BAL fluid correlated well with the number of lymphocytes in BAL fluid from HTLV-I carriers ($r = 0.58$, $p<0.05$, fig 2A), CD4+ cells ($r = 0.77$, $p<0.05$, fig 2B), and CD8+ cells ($r = 0.83$, $p<0.05$, fig 2C).

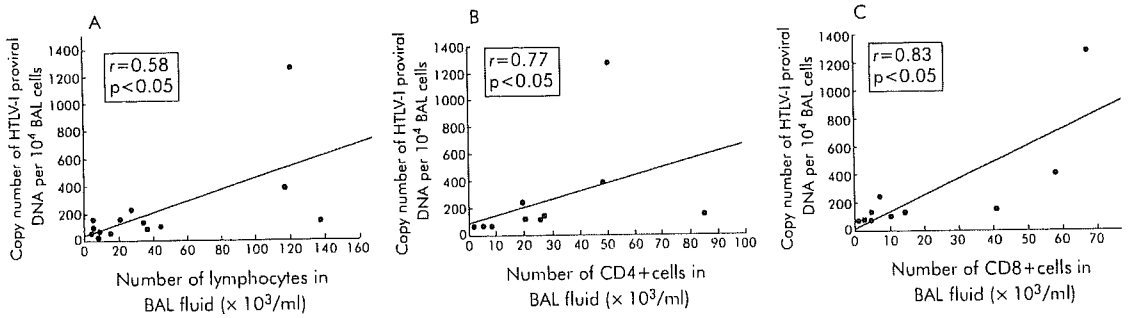

Figure 2 Correlation between (A) the number of lymphocytes in bronchoalveolar lavage (BAL) fluid and the copy number of HTLV-I proviral DNA per 10^4 BAL cells from HTLV-I carriers; (B) the number of CD4+ cells in BAL fluid and the copy number of HTLV-I proviral DNA per 10^4 BAL cells from HTLV-I carriers; and (C) the number of CD8+ cells in BAL fluid and the copy number of HTLV-I proviral DNA per 10^4 BAL cells from HTLV-I carriers. Statistical analyses were performed using Spearman rank correlation. HTLV-I, human T lymphotropic virus type I.

Follow up of asymptomatic HTLV-I carriers with bronchoalveolar lymphocytosis

Three AHCs (subjects 5, 10 and 13) with bronchoalveolar lymphocytosis (>18% lymphocytes in BAL fluid) were available for follow up evaluation of clinical and radiographic variables for 5–10 years. A further individual (subject 9) dropped out 1 year after the study. None of these four individuals showed overt respiratory illness and chest radiographic findings remained normal during the follow up period. They did not develop adult T cell leukaemia, HAM/TSP, or other HTLV-I associated disorders during this time.

DISCUSSION

The major findings of this study are: (1) bronchoalveolar lymphocytosis (>18% lymphocytes in BAL fluid) was observed in 11 of 30 HTLV-I carriers without HAM/TSP or HTLV-I uveitis (36.7%), although there was no significant difference between HTLV-I carriers and control subjects; (2) the number of lymphocytes in the BAL fluid of HTLV-I carriers was significantly correlated with the HTLV-I proviral DNA load in PBMCs; and (3) the HTLV-I proviral DNA load in the BAL fluid was significantly correlated with the number of lymphocytes, CD4+ cells and CD8+ cells in the BAL fluid of HTLV-I carriers.

Recent studies have indicated that immunological dysfunction related to the increased HTLV-I proviral load may be involved in the pathogenesis of HAM/TSP and HTLV-I uveitis.[13 16 19–23] However, some studies have shown that these immunological and virological findings are also present in some HTLV-I carriers including relatives of subjects with HAM/TSP and AHCs who have not developed HAM/TSP or HTLV-I uveitis.[13 16 20] The genetic background may be implicated in the HTLV-I proviral load and immunological dysfunction in HTLV-I carriers.[21 24] These reports suggest that HTLV-I carriers consist of groups of individuals of different genetic backgrounds with various amounts of HTLV-I proviral DNA in PBMCs. Our results showed that the HTLV-I proviral load is high in a subset of HTLV-I carriers without HAM/TSP or HTLV-I uveitis and that the increased HTLV-I proviral load correlates well with bronchoalveolar lymphocytosis in HTLV-I carriers. The present findings, together with those of previous studies, suggest that an increased HTLV-I proviral load may lead to certain systemic conditions including bronchoalveolar lymphocytosis in HTLV-I carriers.

In addition to the genetic background, direct or indirect mechanisms induced by inflammatory conditions may have influenced the HTLV-I proviral load in PBMCs of some SHCs with chronic inflammatory diseases of the respiratory tract in this study, as occurs in HIV-1 infection.[25]

The HTLV-I proviral load in BAL fluid appeared to be related to the proportion of lymphocytes in the BAL fluid of HTLV-I carriers, as reported previously.[26] Interestingly, the HTLV-I proviral load in the BAL fluid correlated with the number of CD8+ cells as well as with the number of CD4+ cells in HTLV-I carriers, even though CD4+ cells are thought to be preferentially infected by HTLV-I.[27] Our findings may be consistent with more recent observations of the tropism of HTLV-I to CD8+ lymphocytes.[28 29] Further investigations are needed to determine which T cell subsets are predominantly infected with HTLV-I in the lungs of HTLV-I carriers.

A few studies have described some AHCs with bronchoalveolar lymphocytosis who did not develop HAM/TSP and HTLV-I uveitis.[30 31] In the present study pulmonary involvement was subclinical in AHCs with bronchoalveolar lymphocytosis, similar to the findings of these previous studies. Furthermore, follow up studies suggested that the clinical development of bronchoalveolar lymphocytosis may be delayed in HTLV-I carriers.

HTLV-I infected lymphocyte(s) commonly exist in the lower respiratory tract of HTLV-I seropositive individuals, as shown in the PCR study of BAL cells. This suggests that factors other than the presence of HTLV-I in the lung—such as a systemic increase in the HTLV-I proviral load, as found in this study—may be necessary for excessive accumulation of lymphocytes in the lung. The mechanisms by which an increased HTLV-I proviral load affects pulmonary involvement in HTLV-I carriers remains to be fully clarified. One possible mechanism is that the increased number of HTLV-I infected cells enhances the probability that infected cells will enter the target organs,[31 32] resulting in a local inflammatory response. However, the frequency of HTLV-I specific cytotoxic T lymphocytes[22 23 33] related to the increased amount of the virus might be involved in the pathogenesis, as has been shown in lung disorders of patients with HIV infection.[25 34]

In conclusion, we have shown that pulmonary lymphocytic infiltration can occur in a subset of HTLV-I carriers who have not developed HAM/TSP or HTLV-I uveitis. This pulmonary involvement may be associated with an increased amount of HTLV-I proviral DNA in peripheral blood.

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LUNG ALERT

Some asthma genotypes may not respond to β_2 agonists

▲ Israel E, Chinchilli VM, Ford JG, *et al.* Use of regularly scheduled albuterol treatment in asthma: genotype-stratified, randomised, placebo-controlled cross-over trial. *Lancet* 2004;**364**:1505–12

This was a prospective crossover trial comparing the use of salbutamol with placebo in 78 mild asthmatics (diagnosed by chest physician, only using inhaled β agonist <56 puffs/week, FEV_1 >70%) aged 18–55 years. 50% had a genetic polymorphism resulting in homozygosity for arginine (Arg/Arg) at amino acid residue number 16 of the β_2 agonist receptor instead of glycine (Gly/Gly), as in the other half. Each patient was matched with a patient from the other group by FEV_1.

Following a 6 week run in period using a placebo metered dose inhaler (two puffs qds; rescue medication ipratropium inhaler), each pair was randomised to receive either active salbutamol (90 μg) or placebo (two puffs qds) for 16 weeks followed by an 8 week run out period using placebo and then crossed over. In the Gly/Gly group there was no change in pre-inhaler morning peak expiratory flow rate (PEFR) with placebo but an increase in PEFR with salbutamol producing a difference of 14 l/min ($p<0.05$). In the Arg/Arg group the reverse occurred with a difference of −10 l/min ($p<0.05$). This group also needed to use their ipratropium inhaler more, which did produce an increase in PEFR. Similar results were seen in FEV_1, symptom scores, and rescue inhaler use.

It appears that Gly/Gly patients respond to salbutamol while those with Arg/Arg seem to get better when salbutamol is withdrawn. It may be that the latter group actually responds to ipratropium. A longer treatment trial is needed with more patients with more severe asthma and with other genetic polymorphisms, using other β_2 agonists, to determine if reliever strategies excluding salbutamol are more suitable for Arg/Arg patients.

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Differences in viral and host genetic risk factors for development of human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis between Iranian and Japanese HTLV-1-infected individuals

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Human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a neurological disease observed only in 1–2 % of infected individuals. HTLV-1 provirus load, certain HLA alleles and HTLV-1 *tax* subgroups are reported to be associated with different levels of risk for HAM/TSP in Kagoshima, Japan. Here, it was determined whether these risk factors were also valid for HTLV-1-infected individuals in Mashhad in northeastern Iran, another region of endemic HTLV-1 infection. In Iranian HTLV-1-infected individuals ($n=132$, 58 HAM/TSP patients and 74 seropositive asymptomatic carriers), although HLA-DRB1*0101 was associated with disease susceptibility in the absence of HLA-A*02 ($P=0{\cdot}038$; odds ratio $=2{\cdot}71$) as observed in Kagoshima, HLA-A*02 and HLA-Cw*08 had no effect on either the risk of developing HAM/TSP or HTLV-1 provirus load. All Iranian subjects possessed *tax* subgroup A sequences, and the protective effects of HLA-A*02 were observed only in Kagoshima subjects with *tax* subgroup B but not in those with *tax* subgroup A. Both the prevalence of HTLV-1 subgroups and the host genetic background may explain the different risks levels for HAM/TSP development in these two populations.

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INTRODUCTION

Human T-cell lymphotropic virus type 1 (HTLV-1) (Poiesz *et al.*, 1980; Yoshida *et al.*, 1982) is a causative agent of adult T-cell leukaemia (Hinuma *et al.*, 1981; Yoshida *et al.*, 1984) and the chronic neurodegenerative disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain *et al.*, 1985; Osame *et al.*, 1986). Only a minority of HTLV-1-infected individuals develop HAM/TSP, and most infected individuals remain healthy throughout their lives. A previous seroepidemiological survey in Kyushu Island, in southwestern Japan, where Kagoshima prefecture is located, estimated the incidence of HAM/TSP among HTLV-1-infected persons at $3{\cdot}1 \times 10^{-5}$ cases per year; assuming a lifespan of 75 years, the lifetime incidence is therefore approximately 0·25 % (Kaplan *et al.*, 1990). In HAM/TSP patients from Kagoshima, the median provirus load in peripheral blood mononuclear cells (PBMCs) is more than ten times higher than HTLV-1-seropositive asymptomatic carriers (HCs) and high provirus load is also associated with an increased risk of progression to disease (Nagai *et al.*, 1998). HTLV-1 provirus load has been correlated with progression of motor disability (Takenouchi *et al.*, 2003) and the risk of sexual transmission of HTLV-1 (Kaplan *et al.*, 1996). Thus, HTLV-1 provirus load is an important correlate of virus transmission as well as disease progression. A previous study

indicated that the provirus load in PBMCs from HCs in genetic relatives of patients with HAM/TSP in Kagoshima was significantly higher than that of non-HAM/TSP-related HCs, suggesting the importance of genetic background for developing HAM/TSP (Nagai *et al.*, 1998). In the Kagoshima population, an association between HLA-DRB1*0101, HLA-B*5401, HLA-A*02 and HLA-Cw*08 and the outcome of HTLV-1 infection has been reported, where HLA-A*02 and HLA-Cw*08 genes were each independently associated with a lower HTLV-1 provirus load and with protection from HAM/TSP, whereas HLA-DRB1*0101 and HLA-B*5401 were associated with an increased susceptibility to HAM/TSP (Jeffery *et al.*, 1999, 2000). The association of HLA-DRB1*0101 with disease susceptibility was only evident in the absence of the protective effect of HLA-A*02 (Jeffery *et al.*, 1999). These results are consistent with the hypothesis that a strong class I-restricted T-cell response is beneficial (Bangham, 2000). In another study, an association between HTLV-1 *tax* gene sequence variation and the risk of HAM/TSP was reported (Furukawa *et al.*, 2000). The *tax* subgroup A was more frequently observed in HAM/TSP patients than in HCs and this effect was independent of HLA-A*02. These reports suggested that both host genetic factors and HTLV-1 subgroup independently play a part in determining the risk of developing HAM/TSP.

HTLV-1 is also endemic in the Caribbean Basin (Blattner *et al.*, 1982), Africa (Biggar *et al.*, 1984), South America (Zamora *et al.*, 1990; Cartier *et al.*, 1993; Zaninovic *et al.*, 1994) and the Melanesian islands (Yanagihara *et al.*, 1990). The city of Mashhad in northeastern Iran has also been reported as an endemic centre for HTLV-1 (Safai *et al.*, 1996). In a recent study, the prevalence of HTLV-I infection was reported to be 0·77 % among blood-bank donors of Mashhad (Abbaszadegan *et al.*, 2003), but the prevalence and incidence of HAM/TSP are unknown in this population. Since there has been no report to compare the genetic risk factors for HAM/TSP among different ethnic populations, it was interesting to study whether genetic risk factors found in Kagoshima, Japan, were also valid for HAM/TSP development in the Mashhadi Iranian population. We therefore analysed the HTLV-1 provirus load, HTLV-1 *tax* subgroup and the allele frequencies of HLA-A*02, HLA-B*5401, HLA-Cw*08 and HLA-DRB1*0101 in Iranian HTLV-1-infected individuals using the same methods and techniques that were used in the Kagoshima studies (Nagai *et al.*, 1998; Jeffery *et al.*, 1999, 2000). The effect of host genetic factors and HTLV-1 *tax* subgroups on the risk of HAM/TSP development in different ethnic groups is discussed.

METHODS

Study populations. Peripheral blood samples were studied from 58 Iranian patients with HAM/TSP and 74 HCs from blood donors of the Blood Transfusion Center in the city of Mashhad and Neyshabour, both located in HTLV-1-endemic northeastern Iran. The study population from Kagoshima consisted of 222 patients with HAM/TSP and 184 HCs, all of whom were enrolled in the previous Kagoshima studies (Nagai *et al.*, 1998; Jeffery *et al.*, 1999, 2000; Furukawa *et al.*, 2000). The diagnosis of HAM/TSP was made according to the World Health Organization diagnostic criteria (Osame, 1990). Informed consent was obtained from all patients. This research was approved by the institutional review boards of the authors' institutions.

DNA preparation. All Japanese and Iranian blood samples were taken by vacuum tube pre-filled with the anticoagulant EDTA. Genomic DNA extraction procedures were different for each population. In the case of Kagoshima samples, fresh PBMCs were isolated by Histopaque-1077 (Sigma) density-gradient centrifugation and genomic DNA was extracted using a QIAamp Blood kit (Qiagen). For Iranian samples, for economical and technical reasons, fresh blood specimens were frozen immediately after collection and frozen whole-blood samples were transported to Kagoshima University on dry ice. Genomic DNA of nucleated blood cells was isolated from whole blood in Kagoshima University using the PureGene DNA Purification kit (Gentra Systems).

Provirus load measurement. To assay the HTLV-1 provirus load, we carried out a quantitative PCR using ABI Prism 7700 (PE Applied Biosystems) with 100 ng genomic DNA (equivalent to approx. 10^4 cells) from PBMCs (for Kagoshima samples) or nucleated blood cells (for Iranian samples) as reported previously (Nagai *et al.*, 1998). Using β-actin as an internal control, the amount of HTLV-1 provirus DNA was calculated using the following formula: copy number of HTLV-1 *tax* per 10^4 PBMCs (for Japanese samples) or nucleated blood cells (for Iranian samples) $=[(\text{copy number of } tax)/(\text{copy number of } \beta\text{-actin}/2)] \times 10^4$. All samples were tested in triplicate. The lower limit of detection was one copy of HTLV-1 *tax* per 10^4 PBMCs. In this study, we used the previously analysed provirus load data of Kagoshima samples from our database (Nagai *et al.*, 1998). All Iranian samples and some randomly selected Kagoshima samples were analysed using the same kit (AmpliTaq Gold and TaqMan probe; PE Applied Biosystems) and machine (ABI Prism 7700) at the same time. The same standard DNA for *tax* and β-actin was used throughout the study and there was no discrepancy between old and new data (not shown).

Sequencing of the HTLV-1 *tax* gene. Randomly selected Iranian samples from 10 HAM/TSP patients and 10 HCs were sequenced over almost the entire HTLV-1 *tax* gene (nt 7295–8356, nucleotide numbers correspond to those of the prototypic strain, ATK-1; Seiki *et al.*, 1983). PCR was done on extracted DNA to amplify provirus DNA, and nucleotide sequences were determined by direct sequencing in both directions. We amplified 100 ng DNA in 35 cycles of PCR, using an expanded high-fidelity PCR system (Boehringer Mannheim) and 1 μM primers ($PXO1^+$, 5′-TCGAAACAGCCCTGCAGATA-3′, nt 7257–7276, and $PXO2^+$, 5′-TGAGCTTATGATTTGTCTTCA-3′, nt 8447–8467). Each PCR cycle consisted of denaturation at 94 °C for 60 s, annealing at 58 °C for 75 s, extension at 72 °C for 90 s and a final extension at 72 °C for 10 min. Amplified DNA products were purified using a purification kit (QIAquick; Qiagen) and 0·1 μg PCR product was sequenced with a dye terminator DNA sequencing kit (Applied Biosystems) with 3·2 pmol each primer [$PXI1^+$, 5′-ATACAAAGTTAACCATGCTT-3′, nt 7274–7293; $PXI2^+$, 5′-GGCCATGCGCAAATACTCCC-3′, nt 7618–7637; $PXI3^+$, 5′-TTCCGTTCCACTCAACCCTC-3′, nt 8001–8020; $PXI1^-$, 5′-GGGTTCCATGTATCCATTTC-3′, nt 7644–7663, $PXI2^-$, 5′-GTCCAAATAAGGCCTGGAGT-3′, nt 8024–8043; and $PXI3^-$, 5′-AGACGTCAGAGCCTTAGTCT-3′, nt 8374–8393] in an automatic DNA sequencer (model 377; Applied Biosystems).

Restriction fragment length polymorphism (RFLP) analysis of the HTLV-1 *tax* gene. To determine the HTLV-1 *tax* gene subgroup (*tax* A or B) in Iranian samples, we carried out a PCR-RFLP

analysis as previously described (Furukawa *et al.*, 2000). For RFLP analysis, 4 μl PCR product was digested with 5 U *Acc*II (Takara) in 10 μl total volume at 37 °C for 1 h followed by electrophoresis on 2 % Nusieve agarose gel. The previously analysed *tax* subgroup data of Kagoshima samples (Furukawa *et al.*, 2000) were extracted from our database. Positive and negative controls of known Japanese samples of *tax* gene subgroups A and B, which were confirmed by direct sequence analysis, were included in all experiments.

HLA typing. PCR sequence-specific primer reactions were performed to detect HLA-A*02, HLA-B*5401, HLA-Cw*08 and HLA-DRB1*0101 as previously described (Bunce *et al.*, 1995; Olerup & Zetterquist, 1992). We used previously analysed HLA data of Kagoshima samples from our database (Jeffery *et al.*, 1999, 2000).

Statistical analysis. Statistical analysis was performed using the SPSS for Windows release 7.0, run on an IBM-compatible computer (Analytical Software, version 7). The χ^2 test, the Mann–Whitney U test and the odds ratio (OR) were used for statistical analysis. Values of $P<0{\cdot}05$ were considered statistically significant.

RESULTS

Differences in HTLV-1 provirus load between HAM/TSP patients and asymptomatic carriers is significantly lower in Iranian HTLV-1-infected individuals than in Japanese

We used the previously analysed provirus load data of Kagoshima samples from our database (Nagai *et al.*, 1998); all Iranian samples were newly analysed. The median age of HAM/TSP patients in both Kagoshima (57·3 years, range 15–80 years, 70·4 % female) and Iran (49·7 years, range 24–80 years, 72·1 % female) was greater than that of HCs in Kagoshima (39·4 years, range 16–64 years, 52·7 % female) and Iran (41·4 years, range 22–73 years, 38·3 % female), respectively. There was no significant difference in age between the control groups (HCs) of the two populations. All HCs in each group originated from unrelated blood donors. Since we extracted Japanese genomic DNA samples from PBMCs but Iranian samples from whole blood, direct comparison of HTLV-1 provirus load between the two populations was inappropriate. Since the main target of HTLV-1 infection is human T cells, whole blood-derived DNA contains more uninfected nucleated cells than PBMCs, and therefore the provirus load data in Iranians was likely to be underestimated if we used β-actin as an internal control. Thus, we compared the HTLV-1 provirus load between HAM/TSP patients and asymptomatic carriers within each population. As shown in Fig. 1, although the HTLV-1 provirus load of Iranian HAM/TSP patients was significantly higher than that of Iranian HCs ($P=0{\cdot}009$, Mann–Whitney U test), as reported in Japanese patients (Nagai *et al.*, 1998), the differences in median provirus load between Iranian HAM/TSP patients and HCs (twofold greater in the HAM/TSP patients than in the HCs) was much smaller than that of Japanese subjects (13-fold). Interestingly, although provirus load data were probably underestimated in Iranian samples compared with Japanese samples, the HTLV-1 provirus load in

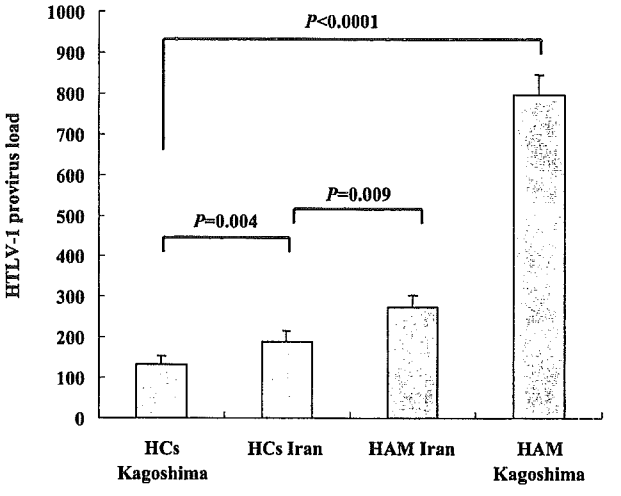

Fig. 1. HTLV-1 provirus load of Japanese and Iranian HTLV-1-infected individuals. Mean HTLV-1 copy numbers per 10^4 PBMCs for Japanese samples and per 10^4 nucleated cells for Iranian samples determined by quantitative PCR are shown. The HTLV-1 provirus load of Iranian HAM/TSP patients was significantly higher than that of Iranian HCs ($P=0{\cdot}009$, Mann–Whitney U test). The difference in median provirus load between Iranian HAM/TSP patients and HCs was much smaller than that of Japanese (Kagoshima) subjects, since HTLV-I provirus load in Iranian HCs is significantly higher than in Japanese HCs ($P=0{\cdot}004$). Error bars indicate SEM.

Iranian HCs was still significantly higher than that of Japanese HCs ($P=0{\cdot}004$, Mann–Whitney U test).

HLA-A*02 and HLA-Cw*08 are not associated with a lower risk of HAM/TSP and a lower provirus load in Iranian HTLV-1-infected individuals

To examine whether the previously reported associations between class I and class II HLA alleles and HAM/TSP prevalence in Kagoshima was also valid for HAM/TSP development in the Iranian population, we genotyped HLA-DRB1*0101 and HLA-A*02, HLA-B*5401 and HLA-Cw*08 by PCR-based DNA typing in 132 Iranian HTLV-1-infected individuals (58 HAM/TSP and 74 HCs). All Japanese HLA data had been previously analysed and were extracted from our database (Jeffery *et al.*, 1999, 2000). As shown in Table 1, the genotype frequency of HLA-A*02 and HLA-Cw*08 in Kagoshima subjects was significantly lower among the cases of HAM/TSP compared with HCs ($P=0{\cdot}0006$ and $0{\cdot}0196$, respectively). In contrast, the genotype frequency of HLA-A*02 and HLA-Cw*08 was not significantly different between Iranian HAM/TSP and HCs ($P=0{\cdot}346$ and $0{\cdot}940$, respectively). Also, whereas HLA-A*02 and HLA-Cw*08 were associated with a lower median provirus load in Kagoshima subjects ($P=0{\cdot}0003$ for A*02 and $P=0{\cdot}009$ for HLA-Cw*08; Mann–Whitney U test), this effect was not observed in Iranian subjects

Table 1. HLA-A*02 and HLA-Cw*08 are not associated with a lower risk of HAM/TSP in Iranian HTLV-1-infected individuals

Population (no. HAM/HCs)	HLA allele	HAM/TSP	HCs	χ^{2}*	P	OR†	95% CI
Iranian (58/74)	HLA-A*02^{+}	21 (36·2%)	20 (27·0%)	0·887	0·346	1·53	0·73–3·22
	HLA-A*02^{-}	37 (63·8%)	54 (73·0%)				
Japanese (222/184)‡	HLA-A*02^{+}	67 (30·2%)	87 (47·3%)	11·784	0·0006	0·48	0·32–0·72
	HLA-A*02^{-}	155 (69·8%)	97 (52·7%)				
Iranian (58/74)	HLA-Cw*08^{+}	9 (15·5%)	10 (13·5%)	0·006	0·940	1·18	0·44–3·11
	HLA-Cw*08^{-}	49 (84·5%)	64 (86·5%)				
Japanese (222/184)‡	HLA-Cw*08^{+}	24 (10·8%)	36 (19·6%)	5·45	0·0196	0·50	0·29–0·87
	HLA-Cw*08^{-}	198 (89·2%)	148 (80·4%)				

*With Yates correction.

†OR used the approximation of Woolf (1955).

‡Japanese data were extracted from a database from previous analyses (Jeffery *et al.*, 1999, 2000).

($P=0·071$ for A*02 and $P=0·75$ for HLA-Cw*08; Mann–Whitney U test; Table 2), indicating that a protective effect of HLA-A*02 and HLA-Cw*08 was not observed in Iranian HTLV-1-infected individuals. As expected, HLA-B*5401, which is known to be almost exclusively found in East Asian populations, was not found in the Iranian subjects analysed.

HLA-DRB1*0101 increases the odds of HAM/TSP development in both Japanese and Iranian HLA-A*02-negative, but not in HLA-A*02-positive, HTLV-1-infected individuals

In contrast to HLA-A*02, HLA-DRB1*0101 was associated with susceptibility to HAM/TSP in both Japanese ($P=0·049$) and Iranian ($P=0·035$) populations (Table 3). This effect was observed only in the HLA-A*02-negative subjects but not in the HLA-A*02-positive subjects in both populations (Table 3). Although possession of HLA-DRB1*0101 was associated with a significantly lower provirus load in the Japanese HAM/TSP patients (Table 4, $P=0·024$) but not in HCs, HLA-DRB1*0101 was not associated with a difference in the provirus load in the Iranian HTLV-1-infected HAM/TSP patients and HCs (Table 4).

All Iranian HTLV-1 isolates have 10 nt substitutions in the *tax* region including all the *tax* subgroup A substitutions

Based on the LTR gene sequence, HTLV-1 can be classified into three types: Melanesian, Central African and cosmopolitan types, while cosmopolitan types can be further classified into subtypes A, B and C (Miura *et al.*, 1994). There are two distinct subtypes in Japan; the most frequently observed (nearly 80%) Japanese subtype belongs to cosmopolitan subtype B, while a minor subtype (20%), which seems to cluster in the southern islands of Kyushu and the Ryukyu Islands, belongs to cosmopolitan subtype A. A previous report suggested that, although Mashhadi HTLV-1 isolates belonged to cosmopolitan subtype A, this strain formed a tight cluster that was distinct from the other isolates of cosmopolitan subtype A from Japan, India, the Caribbean Basin and South America (Yamashita

Table 2. HLA-A*02 and HLA-Cw*08 are not associated with a lower provirus load in Iranian HTLV-1-infected individuals

Population	HLA allele	Provirus load (mean ± SE)*	Provirus load (median)*	No. subjects	P†
Iranian	HLA-A*02^{+}	262·1 ± 34·5	190·0	41	0·071
	HLA-A*02^{-}	209·6 ± 24·9	120·0	91	
Japanese‡	HLA-A*02^{+}	366·8 ± 43·4	118·5	156	0·0003
	HLA-A*02^{-}	525·6 ± 41·5	266·0	250	
Iranian	HLA-Cw*08^{+}	198·2 ± 42·8	131·0	19	0·75
	HLA-Cw*08^{-}	233·6 ± 22·9	147·0	113	
Japanese‡	HLA-Cw*08^{+}	300·7 ± 56·4	120·0	60	0·009
	HLA-Cw*08^{-}	492·0 ± 34·5	234·0	346	

*Provirus load is the HTLV-1 *tax* copy number per 10^{4} PBMCs for Japanese samples and per 10^{4} nucleated cells for Iranian samples by quantitative PCR.

†Two-tailed Mann–Whitney U test.

‡Japanese data were extracted from a database of previous analyses (Nagai *et al.*, 1998; Jeffery *et al.*, 1999, 2000).

Table 3. HLA-DRB1*0101 increases the odds of HAM/TSP development in Japanese and Iranian HLA-A*02-negative, but not in HLA-A*02-positive, HTLV-1-infected individuals

Population	Subjects	HAM/TSP (*n*)		HCs (*n*)		χ^{2}*	*P*	OR†	95% CI
		DRB1^{+}	DRB1^{-}	DRB1^{+}	DRB1^{-}				
Iranian	All	18	40	12	62	3·3	0·035	2·33	1·01–5·34
	A*02^{-}	13	24	9	45	3·1	0·038	2·71	1·01–7·24
	A*02^{+}	5	16	3	17	0·1	0·376	1·77	0·36–8·65
Japanese‡	All	34	161	20	163	2·8	0·049	1·72	0·95–3·12
	A*02^{-}	27	107	10	83	2·9	0·044	2·09	0·96–4·57
	A*02^{+}	7	54	10	80	0·005	0·47	1·03	0·37–2·89

*Reported as one-tailed with Yates correction.

†OR used the approximation of Woolf (1955).

‡Japanese data were extracted from a database of previous analyses (Jeffery *et al.*, 1999).

Table 4. HLA-DRB1*0101 associated with lower HTLV-1 provirus load in Japanese but not in Iranian HAM/TSP patients

The DRB1-positive Japanese HAM/TSP patients developed HAM/TSP with a significantly lower provirus load than DRB1-negative HAM/TSP patients, but this effect was not observed in Iranian HAM/TSP patients.

Population	HLA allele	HAM/TSP			HCs		
		Median provirus load*	No. subjects	*P*†	Median provirus load*	No. subjects	*P*†
Iranian	DRB1^{-}	193·0	40	0·31	115·0	62	0·34
	DRB1^{+}	357·0	18		104·0	12	
Japanese‡	DRB1^{-}	602·6	161	0·024	34·7	163	0·33
	DRB1^{+}	331·1	34		49·0	20	

*Provirus load is HTLV-1 *tax* copy number per 10^{4} PBMCs for Japanese samples and per 10^{4} nucleated cells for Iranian samples by quantitative PCR.

†*P* level reported using two-tailed Mann–Whitney U test.

‡Japanese data were extracted from the database of previous analyses (Nagai *et al.*, 1998; Jeffery *et al.*, 1999).

et al., 1995). A previous report indicated that the *tax* subgroup A was more frequently observed in HAM/TSP patients in the Kagoshima cohort and that this effect was independent of HLA-A*02 (Furukawa *et al.*, 2000). The higher HAM/TSP risk *tax* subgroup A corresponds to the cosmopolitan subtype A, and the lower HAM/TSP risk *tax* subgroup B corresponds to the cosmopolitan subtype B according to the LTR sequence (Furukawa *et al.*, 2000). We sequenced almost the entire *tax* region of HTLV-1 provirus (nt 7295–8356) from 20 different Iranian subjects (10 HAM/TSP and 10 HCs) by direct sequencing in both directions. As shown in Table 5, all Iranian HTLV-1 sequences (EMBL/GenBank/DDBJ accession no. AB181224) differed at 10 nt compared with the Japanese prototypic

Table 5. Nucleotide variations specific to Iranian HTLV-1

Amino acid changes in *tax* A resulting from the nucleotide substitution are shown. Nucleotide numbers correspond to those of the prototypic strain, ATK-1 (Seiki *et al.*, 1983). N, No change.

Subgroup	Nucleotide variation (nucleotide position and amino acid change)									
	7622 M→V	7811 I→V	7855 N	7897 N	7959 A→V	7991 N→H	8208 S→N	8313 G→E	8314 N	8344 N
ATK-1 (=*tax* B)	A	A	T	C	C	A	G	G	C	A
tax A				T	T		A			C
Iranian *tax*	G	G	C	T	T	C	A	A	G	C

Table 6. HLA-A*02 is associated with a lower risk of HAM/TSP development only in *tax* subgroup B subjects in Kagoshima

Japanese data were extracted from a database of previous analyses (Jeffery *et al.*, 1999; Furukawa *et al.*, 2000).

tax subgroup	HLA allele	HAM/TSP	HCs	χ^{2}*	*P*	OR†	95% CI
tax A	HLA-A*02^{+}	16 (57%)	6 (60%)	0·047	0·829	0·89	0·20–3·87
	HLA-A*02^{-}	12 (43%)	4 (40%)				
tax B	HLA-A*02^{+}	51 (26%)	81 (47%)	15·5	<0·0001	0·41	0·26–0·63
	HLA-A*02^{-}	143 (74%)	93 (53%)				

*With Yates correction.
†OR used the approximation of Woolf (1955).

ATK-1 strain (Seiki *et al.*, 1983). Among these, nt 7897, 7959, 8208 and 8344 were exactly the same as those in *tax* subgroup A. In addition to these four residues, the Iranian *tax* sequences had 6 nt differences, which encoded four additional amino acid differences from Japanese *tax* subgroup A. We further performed PCR-RFLP analysis to determine the HTLV-1 *tax* subgroup (*tax* A or B) of all of the remaining Iranian samples and found that all Iranian HTLV-1 isolates had *tax* subgroup A substitutions.

HLA-A*02 is associated with a lower risk of HAM/TSP and a lower provirus load only in HTLV-1-infected individuals with *tax* subgroup B in Kagoshima subjects

As the majority of HTLV-1 isolates observed in the Kagoshima population were *tax* subgroup B, we examined further whether the effect of HLA-A*02 on the risk of HAM/TSP and HTLV-1 provirus load was observed only in HTLV-1 *tax* subgroup B-infected individuals in Kagoshima subjects. Japanese *tax* subgroup data were extracted from our existing database (Furukawa *et al.*, 2000). As shown in Table 6, the effects of HLA-A*02 on the risk of HAM/TSP and provirus load were not observed in HTLV-1 *tax* subgroup A-infected subjects in Kagoshima. We next sought a possible interaction between HLA-A*02 and HTLV-1 provirus load among HTLV-1 *tax* subgroup A-infected subjects in Kagoshima (Table 7). HLA-A*02 was associated with a lower provirus load only in the *tax* subgroup B subjects in Kagoshima, but not in the *tax* subgroup A subjects in either Japan or Iran.

DISCUSSION

Currently, several different approaches including family-based linkage and population-based case–control studies have been used to identify genetic susceptibility to numerous infectious pathogens such as malaria, mycobacteria, hepatitis viruses and human immunodeficiency virus (Hill, 1998). The candidate gene approach (case–control studies) can only utilize known genes and will not identify unknown genes, but genome-wide linkage studies have less power than candidate gene studies to pick up genes that have only a small or moderate effect on disease risk; therefore the two approaches are complementary. Although our Kagoshima cohort of HAM/TSP is the world's largest, only 300 HAM/TSP patients were available for analysis. Also, extensive studies in one ethnic population may not disclose the marker-disease distance or exclude a possible spurious association due to admixture. Studies in different ethnic populations may thus provide useful information about marker-disease distance, as well as confirming the reliability of results from our previous association studies. In this study, we compared the risk factors for developing HAM/TSP in two ethnic groups living in quite different environments, namely, Kagoshima in southwest Japan and Mashhad in northeast Iran. It is

Table 7. HLA-A*02 is associated with a lower provirus load only in *tax* subgroup B subjects in Kagoshima

Japanese data were extracted from a database of previous analyses (Nagai *et al.*, 1998; Jeffery *et al.*, 1999; Furukawa *et al.*, 2000).

tax subgroup	HLA allele	Provirus load (mean ± SE)*	Provirus load (median)*	No. subjects	*P*†
tax A	HLA-A*02^{+}	635·0 ± 169·3	389·0	22	0·98
	HLA-A*02^{-}	586·4 ± 164·9	356·5	16	
tax B	HLA-A*02^{+}	328·5 ± 41·6	99·0	132	0·0001
	HLA-A*02^{-}	520·0 ± 42·7	266·0	236	

*Provirus load is the HTLV-1 *tax* copy number per 10^{4} PBMCs by quantitative PCR.
†Two-tailed Mann–Whitney U test.