A. 研究目的
一般的に統計学を用いた研究仮説の検証は大標本理論をもとにした要約統計量を求めることにより群間比較や要因分析により行われる。DNA メチル化データの統計学的解析方法においても、広く適用されているものはこの大標本理論に基づいたものがほとんどである。しかしながら、網羅的ゲノム解析は現在もコストがかかることにより大標本を得ることは難しく、効率的研究デザインおよび小サンプルでの仮説探索が不可欠となる。今回の研究のように In vitro で特に制御された環境下においては実験デザインの工夫により環境要因による分散成分を限りなく小さくしていくことにより効率を上げていくことが可能である。さらに、臨床検体を用いた先行研究より、メチル化状態が真に変化していると考えられる個体あるいは遺伝子に関しては、全体よりかなりはずれた値を示すことが示され、より小さいサンプルサイズにより変化をとらえることの可能性が示されたことを踏まえ、本研究では、a:全ゲノムＤＮＡメチル化測定データの分布に基づく個体間差の検出、b:小サンプルでの複数のバラツキの指標を用いた多群比較法を用いた DNA メチル化感受性遺伝子の検出方法の検討を行うことを目的とする。

B. 研究方法
目的 a および b について用いた実データはすでに出版済みの HIV とリンパ腫との関連を検討したイルミナ４５０K チップにより測定されノーマライゼーション済みの 28 検体分、375639 プローブのデータである。
目的 a および b について用いたシミュレーションデータは、全ゲノムメチル化データが２～３混合ベータ分布に従うことから以下のようないくつかのパラメタを設定して発生させた。
Scenario 1 (two-peaks model): a1=1, b1=12, a2=13, b2=2, w1=0.4, w2=0.6;
Scenario 2 (three-peaks model): a1=7.0, b1=43.6, a2=2.6, b2=3.9, a3=14.0, b3=1.8,w1=0.26, w2=0.31, w3=0.43;
Scenario 3 (four-peaks model): a1=5, b1=70 a2=8, b2=26, a3=45, b3=25, a4=64, b4=6, w1=0.35, w2=0.15, w3=0.16, w4=0.34.
データの発生には R vers.2.15 を用いた。
目的 a については、臨床検体を用いた先行研究データを用いて適切なデータの視覚化および、パラメトリックおよびノンパラメトリックな分布当てはめと形状パラメタの推定のためのプログラムを R を用いて開発した。シミュレーションデータについては真の分布の混合数がわかっているが、実データについては真の混合数が分からないため、データを視覚化した際に観測されるピークの数を数えることで、真の混合数を設定した。尚、観測者によるバイアスを除去するために、ピーク数の数え上げは独立した場所で 3 人によって行われ、多数決によって真の混合数が決定された。3 人とも回答が異なった場合は、判定不能とした。
目的 b については、まず本年度は、2 群比較のみを行うこととした。一般的に適用されている t 検定（群ごとの分散が等しいという仮定のもとで平均値の比較）、ウィルコクソン順位和検定（群ごとの分布形が等しいという仮定のもとでノンパラメトリックな中央値の比較）に加え、ノンパラメトリックな F 検定に対応する Ansari-Bradley 検定（群ごとの分布型が等しいという仮定の下でノンパラメトリックな分散の比較）、および、分布型も分布の代表値も違うことを検出するためのコルモゴロフスミルノフ検定を実データに適用し、選択される候補領域にどのくらい差があるのかを観察した。

C. 研究結果
a 前年度において、多次元 β 分布を各サンプルのメチル化測定データに当てはめて、個体間での分布比較が可能かどうかについて実データおよびシミュレーションデータで検討を行った結果、感度が 70~90%と比較的良好であったが、特異度が 0~50%と低い値であった。そこで、測定の生データは β 分布に従うことが理論的に示されているが、変数変換することで、漸近的に多次元正規分布に従うと考えられるため、サンプ

ルごとに変数変換後多次元正規分布を当てはめ、再度感度・特異度についてプレリミナリーな検討を行った結果、感度・特異度ともにβ分布で検討するよりも高い結果となった。

b　４つの検定手法を NCGM データに適用した結果、たとえば、p 値の小さい順 20 プローブを選択するとした場合、375639 プローブ中 66 プローブが選択された。このうち 12 プローブが二つの手法で選択されていたが、そのうち９プローブはウィルコクソン順位和検定とコルモゴロフスミルノフ検定で選択されていたものであった。ｔ検定やウィルコクソン順位和検定で検出されず、コルモゴロフ検定で検出されたものに関して分布型を検討した結果、コントロール群で比較的標準的な単法性の分布型であるのに、ケース群（今回は HIV 感染群）で２峰性をとっているものなど、分子生物学的には重要そうな差を検出している可能性が示唆された。

D. 考察

a について

混合β分布の当てはめは、ノイズに非常に敏感であること、計算時間がかかることなどから、実用性に低い可能性が示された。多次元正規分布を当てはめることで、個人のメチル化分布の差の検出が可能であることが示されたが、さらに、多次元正規分布に従わないようなノイズの多い分布が実データでは発生することも考えられるため、ノンパラメトリックに核関数で分布を推定し、分布のピークを数え上げることで分布の個人間差を検討する方法についても同様に性能評価を行うこととした。

b について

今後は、どのくらいのサンプルサイズで漸近性が保たれるのか、あるいはある程度分布型が異なる場合においてもｔ検定でも十分検出されるようになるのかなどをシミュレーションデータで検討することも必要と考える。また、検定手法によって検出したい差が異なるので、複合的評価基準を設けることで候補領域を見逃さない手法の提案を行う必要性があると考えられた。

A. 結論

混合β分布の当てはめは、ノイズに非常に敏感であること、計算時間がかかることなどから、実用性に低い可能性が示された。今後実用性の高い別の方法の開発が必要であることが示唆された。

候補領域の絞り込みには、検出したい差に基づいた複数の手法の複合的評価基準を設けることが必要であることが示唆された。

B. 研究発表

(学会発表)

Tanaka N, Kurosawa T, Inaba Y, Toyo-oka L, Yoshida L, Kawasaki Y. Filtering samples based on Beta-Micture model for DNA methylation data Quantified by Bisulphite microarrays. International Biometric Conference 2014. Florence. Italy. July. 2014.

C. 知的財産権の出願・登録状況

無し.

別紙３

厚生労働科学研究費補助金（エイズ対策　研究事業）
分担研究報告書

Vprによるp53不活性化機構

研究分担者　山下　克美　金沢大学医薬保健研究域・准教授

研究要旨　HIV ゲノムにコードされる Vpr 遺伝子の単独発現による DNA 損傷誘発を、ゲノム損傷応答因子 p53 およびその上流の ATR、p53 の下流の CHK1 について、活性化の指標であるリン酸化を、ウエスタンブロットにより検出した。その結果、いずれの因子も活性化されている事が判明した。

A．研究目的

近年、長期にわたる感染者において、腫瘍の発生が報告されている。細胞の癌化においてゲノム不安定化が必須な役割を演じていることは周知の事実である。

本研究の主任研究者である石坂らは、HIV ゲノムコードされる Vpr タンパク質が DNA 損傷を誘発することを発表し、Vpr が HIV 感染細胞におけるゲノム不安定化の誘因となり、ひいては HIV 感染者における腫瘍発生の重要な原因因子である可能性を示した。

本研究ではこの仮説に基づき、Vpr 誘発性のゲノム損傷応答系のエンドポインの一つである、癌抑制遺伝子 p53 の動態を解明することが目的である。その成果を成果を基盤として、p53 の動態を直接的または間接的に抑制する低分子化合物の探索等、HIV 感染細胞における Vpr を標的とした癌化抑制法の開発を目指す。

B．研究方法

本研究では、Vpr 発現モデルとして石坂らによって開発された、テトラサイクリン（Tet）添加により Vpr タンパクが発現される HT1080 細胞（MIT-23 細胞）を使用した。

Tet不含培地で培養したMIT-23細胞に1-5μg/mLのドキシサイクリン（Dox：Tet 誘導体）を添加した。48 時間後に細胞を回収し、検討対象タンパクの発現と DNA 損傷誘発性リン酸化をウエスタンブロット法にて検討した。

検討対象のタンパク質は、p53、p53(pS15)、CHK1(pS345)、p21 および Vpr である。

（倫理面への配慮）

現段階では培養細胞を使用する研究のため、倫理面への配慮は不要である。

C．研究結果

p53 の発現誘導：3μg/mL と 5μg/mL の Dox 処理において、発現上昇が認められたが、それ以下の濃度では検出不能であった。

p53(S15)のリン酸化：p53 の発現と同様に、3μg/mL と 5μg/mL の Dox 処理においてのみリン酸化が検出された。

CHK1(S345)のリン酸化：p53 発現および S15 のリン酸化と同様に、3μg/mL と 5μg/mL の Dox 処理においてのみリン酸化が認められた。

p21 の発現：p21 は p53 の転写ターゲットであるため、p53 発現が認められる Dox dose での発現が期待されたが、発現は確認できなかった。

Vpr の発現：Dox 添加による Vpr の発現は、すべての dose において検出できなかった。

D．考察

本研究では、p53 関連の応答と CHK1 のリン酸化が検出された。p53 の転写標的である p21 の誘導は検出できなかったが、検出に使用した抗体が原因の可能性があり、今後の検討課題である。また、DNA 損傷を惹起する Vpr の発現誘導も検出できていないが、過去の石坂らの実験では Vpr タンパク質が検出されているため、条件検討で問題が解決する可能性が高い。

Vpr の発現に関しては、p53 の発現誘導、p53S15 のリン酸化ならびに CHK1S435 のリン酸化が認められているため、現時点では検出できていないが、発現はされているものと考えられる。

Vpr 誘発性の DNA 損傷リスポンス(DDR:DNA damage response)の一経路として p53 が誘導されることは（本研究では p21 の検出をできなかったが）、p53 の下流の細胞応答が引き起こされていることが強く示唆される。Vpr は DDR のみならず、多様な細胞変化を誘導し、細胞がん化の誘因となる

可能性が示されており、今後は p53 活性化の下流の遺伝子発現の解析等が、Vpr の発がんにおける機能を明らかにする上で重要である。

Vpr は細胞周期において、G2 期から M 期への進行および、M 期の進行遅延を引き起こすことも知られており、これらのイベントが染色体不安定化の原因となることも示されている。一方、DNA 損傷によって G2 から M 期への進行遅延が引き起こされることは周知の事実である。これらの事実は、Vpr が誘発する DNA 損傷が M 期侵入遅延および M 期進行の撹乱の原因である可能性も示唆する。

Vpr 誘発性 DDR としての p53 の動態ならびに、DDR としての細胞周期進行遅延の解明は、AIDS 関連性の悪性腫瘍誘発の分子機序の理解に重要な情報をもたらすことが期待されるため、DDR と細胞周期進行の関連を追求することが今後の重要な課題となる。

E．結論

今年度の研究では、Vpr の発現によって誘発される DDR を検出することを試みた。その結果、p53 の発現誘導、リン酸化、CHK1 の活性化を示すリン酸化等が検出された。

本研究では、p53 の重要な下流因子である p21 の発現が検出できなかった。さらに、Vpr タンパク質も検出できなかったため、Vpr 誘導発現系の改良が求められる。

F．健康危険情報

特になし。

G．研究発表

1. 論文発表

なし。

2. 学会発表

なし。

H．知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得

なし。

2. 実用新案登録

なし。

3. その他

特記すべき事なし。

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kikuchi, T., Iwabu, Y., Tada, T., Kawana-Tachikawa, A., Koga, M., Hosoya, N., Nomura, S., Brumme, Z.L., Jessen, H., Pereyra, F., Piechocka-Trocha, A., Walker, B.D., Iwamoto, A., Tokunaga, K (co-corresponding author), Miura, T.	Anti-APOBEC3G activity of HIV-1 Vif protein is attenuated in elite controllers	J. Virol.	89	4992-5001	2015

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Journals.ASM.org

Anti-APOBEC3G Activity of HIV-1 Vif Protein Is Attenuated in Elite Controllers

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ABSTRACT

HIV-1-infected individuals who control viremia to below the limit of detection without antiviral therapy have been termed elite controllers (EC). Functional attenuation of some HIV-1 proteins has been reported in EC. The HIV-1 accessory protein Vif (virion infectivity factor) enhances viral infectivity through anti-retroviral factor apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G) degradation; however, little is known regarding Vif function in EC. Here, the anti-APOBEC3G activities of clonal, plasma HIV RNA-derived Vif sequences from 46 EC, 46 noncontrollers (NC), and 44 individuals with acute infection (AI) were compared. Vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped viruses were generated by cotransfecting 293T cells with expression plasmids encoding patient-derived Vif, human APOBEC3G, VSV-G, and a *vif/env*-deficient *luciferase*-reporter HIV-1 proviral DNA clone. Viral stocks were used to infect 293T cells, and Vif anti-APOBEC3G activity was quantified in terms of luciferase signal. On average, the anti-APOBEC3G activities of EC-derived Vif sequences (median $\log_{10}$ relative light units [RLU], 4.54 [interquartile range {IQR}, 4.30 to 4.66]) were significantly lower than those of sequences derived from NC (4.75 [4.60 to 4.92], $P < 0.0001$) and AI (4.74 [4.62 to 4.94], $P < 0.0001$). Reduced Vif activities were not associated with particular HLA class I alleles expressed by the host. Vif functional motifs were highly conserved in all patient groups. No single viral polymorphism could explain the reduced anti-APOBEC3G activity of EC-derived Vif, suggesting that various combinations of minor polymorphisms may underlie these effects. These results further support the idea of relative attenuation of viral protein function in EC-derived HIV sequences.

IMPORTANCE

HIV-1 elite controllers (EC) are rare individuals who are able to control plasma viremia to undetectable levels without antiretroviral therapy. Understanding the pathogenesis and mechanisms underpinning this rare phenotype may provide important insights for HIV vaccine design. The EC phenotype is associated with beneficial host immunogenetic factors (such as HLA-B*57) as well as with functions of attenuated viral proteins (e.g., Gag, Pol, and Nef). In this study, we demonstrated that HIV-1 Vif sequences isolated from EC display relative impairments in their ability to counteract the APOBEC3G host restriction factor compared to Vif sequences from normal progressors and acutely infected individuals. This result extends the growing body of evidence demonstrating attenuated HIV-1 protein function in EC and, in particular, supports the idea of the relevance of viral factors in contributing to this rare HIV-1 phenotype.

HIV-1-infected individuals who control viremia to below the limit of detection (<50 RNA copies/ml plasma) without antiretroviral therapy have been termed elite controllers (EC) (1, 2), while those with prolonged survival are known as slow progressors (SP) or long-term nonprogressors (LNTP). Although the mechanisms underlying these protective phenotypes remain incompletely understood, host genetics, innate and adaptive immune responses, and viral sequence variation represent likely contributors (1, 3, 4). Immunologic and host genetic factors associated with slower HIV disease progression include heterozygosity for a 32-bp deletion in the CCR5 gene (5), expression of particular HLA class I alleles (especially HLA-B*57 and B*27) (2, 6–9), ability to mount Gag-specific cytotoxic T lymphocyte (CTL) responses (10–12), quality of HIV-specific CTLs (13) or of $CD4^+$ T lymphocytes (14), and epistatic interactions between HLA-Bw4 and NK cell receptor KIR3DS1 (15). However, these factors incompletely explain elite control.

Viral factors also affect HIV-1 disease progression and/or set-point viral load. For example, deletions in the *nef* gene have been described in some LTNPs (16, 17). Furthermore, reduced entry

Received 4 December 2014 Accepted 30 January 2015
Accepted manuscript posted online 25 February 2015
Citation Kikuchi T, Iwabu Y, Tada T, Kawana-Tachikawa A, Koga M, Hosoya N, Nomura S, Brumme ZL, Jessen H, Pereyra F, Trocha A, Walker BD, Iwamoto A, Tokunaga K, Miura T. 2015. Anti-APOBEC3G activity of HIV-1 Vif protein is attenuated in elite controllers. J Virol 89:4992–5001. doi:10.1128/JVI.03464-14.
Editor: S. R. Ross
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doi:10.1128/JVI.03464-14

efficiency of EC-derived *env* sequences (18), reduced protein functions of *nef* genes (19), and reduced replication capacity of recombinant virus expressing EC-derived *gag* and *pol* sequences (20, 21) have been reported in EC, including at the earliest stages of infection (22). Together, these data support virologic factors as additional determinants of elite control and suggest a potentially important role of genotypic and/or functional characteristics of the transmitted virus in the infection course. However, the contribution of genetic and/or functional properties of other HIV accessory proteins to the controller phenotype remains unknown.

Vif (virion infectivity factor) is an accessory protein that is essential for HIV-1 infectivity in primary $CD4^+$ T lymphocytes (23). This viral protein mediates the degradation of the endogenous antiretroviral factor apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G) in virus-producing cells (24–27). APOBEC3G belongs to the APOBEC family of proteins possessing cytidine deaminase activity (28). In the absence of Vif, APOBEC3G induces a high rate of C-to-U lesions in the first minus strand of cDNA during the process of reverse transcription. This leads to G-to-A hypermutation in the plus-strand DNA, resulting in a potent restriction of viral infectivity (29, 30). Vif inhibits the lethal incorporation of APOBEC3G into virions by targeting it for ubiquitin-mediated degradation in virus-producing cells, via a mechanism involving the assembly of the Cullin5-ElonginB-ElonginC E3 ubiquitin ligase complex (31, 32). Though some studies have reported the presence of mutated or defective *vif* sequences in LTNPs (33–36), the relationship between Vif genotypic/phenotypic variation and HIV disease progression remains incompletely characterized.

In the present study, the anti-APOBEC3G activity of Vif proteins derived from HIV-1-infected elite controllers was compared to the anti-APOBEC3G activity of those from noncontrollers (NC) and from individuals with acute infection (AI). We observed significant attenuation of anti-APOBEC3G activity of Vif proteins derived from EC that did not appear to be attributable to a common single viral genetic defect in these patients.

MATERIALS AND METHODS

Study subjects and plasma collection. The EC, AI, and NC cohorts have been described in detail elsewhere (10, 20, 37, 38). Briefly, EC were defined as having plasma HIV-1 RNA levels of <50 copies/ml in the absence of antiretroviral therapy at least 3 times over a 12-month period (episodes of plasma viremia of up to 1,000 copies/ml were permitted if they were not consecutive and if they represented the minority of all determinations). EC were recruited from outpatient clinics at local Boston hospitals and were referred from providers throughout the United States. Untreated NC, recruited from Boston hospitals, were defined as having plasma HIV-1 RNA levels above 2,000 copies/ml. Untreated AI were enrolled through a private medical clinic (Jessen-Praxis) in Berlin, Germany, and through Massachusetts General Hospital, Boston, MA. AI was defined according to published criteria (Acute Infection, Early Disease Research Program [AIEDRP] sponsored by NIAID) (39, 40). The estimated date of infection of AI was calculated according to the AIEDRP criteria (41). A total of 47 untreated EC (all with <50 copies/ml plasma; median CD4 count, 850 [interquartile range {IQR}, 603 to 1,057] cells/μl), 46 untreated NC (median plasma viral load, 4.89 [IQR, 4.12 to 5.22] $\log_{10}$ copies/ml; median CD4 count, 323 [IQR, 60 to 488] cells/μl), and 44 AI (median plasma viral load, 5.87 [IQR, 5.30 to 6.51] $\log_{10}$ copies/ml; median CD4 count, 453 [IQR, 364 to 650] cells/μl), sampled a median of 39 (IQR, 31 to 59) days after infection, were studied. For 1 EC and for 3 AI, CD4 counts were unavailable at the time of sampling. The study was approved by the Institutional Review Board of Massachusetts General Hospital, and written informed consent was obtained from all participants. Plasma was obtained by standard procedures and stored at −80°C until use.

Viral RNA isolation. For EC, a mean of 19.7 (range, 4.5 to 35.0) ml plasma was centrifuged for 10 min at 1,500 rpm to remove cell debris. Virus was then concentrated by ultracentrifugation at a relative centrifugal force of 124,000 for 2 h using an SW32 Ti rotor (Beckman Coulter, Fullerton, CA). The supernatant was removed, and HIV RNA was extracted from the resulting pellet using a Qiagen viral RNA minikit (Qiagen Inc., Valencia, CA). For NC and AI, viral RNA was isolated from 0.5 ml of plasma. Viral RNA was eluted in 80 μl of DNase- and RNase-free water and stored at −80°C.

PCR amplification. HIV-1 Vif was amplified from extracted plasma HIV RNA by nested reverse transcription-PCR (RT-PCR) using first-round primers spanning the 3′ half of HIV-1 genome (forward, GCATT CCCTACAATCCCCAAAG [HXB2 nucleotides 4648 to 4669]; reverse, GCACTCAAGGCCAAGCTTTATTGAGGC [HXB2 nucleotides 9629 to 9604]), followed by second-round primers incorporating restriction sites for cloning (forward, GG<u>GGTACC</u>ATGGAAAACAGATGGCAGGTG [HXB2 nucleotides 5033 to 5061] [the KpnI restriction site is underlined]; reverse, CTAG<u>TGGCCA</u>TTCATTGTATGGCTCCC [HXB2 nucleotides 5619 to 5593] [the MscI restriction site is underlined]). For all of the ECs and 19 of the NCs, first-round amplicons had previously been generated as part of a published study (38). A SuperScript III one-step RT-PCR system with Platinum *Taq* High Fidelity (catalog no. 12574-030; Invitrogen) and TaKaRa EX *Taq* DNA polymerase, Hot Start version (catalog no. RR006; TaKaRa Bio Inc., Shiga, Japan), were used to generate first-round and second-round amplicons, respectively. Amplification of second-round PCR products was confirmed by agarose gel electrophoresis.

Proviral DNA constructs, plasmids, and vectors. HIV-1 proviral construct pNL4-3, Vif-deficient HIV-1 proviral indicator construct pNL-*Luc-vif*(-)*env*(-), vesicular stomatitis virus glycoprotein (VSV-G) expression vector pHIT/G, and hemagglutinin (HA)-tagged human APOB EC3G expression plasmid pCA-hA3G-HA were described elsewhere (42–45). The GenBank accession number for the human APOBEC3G sequence used for constructing pCA-hA3G-HA is NM_021822.

Vif sequence analysis and cloning. Second-round *vif* amplicons were (directly) bulk sequenced using a 3130xl genetic analyzer (Applied Biosystems). Viral nucleotide sequences were edited using Sequencher 4.8 (Gene Codes Corporation). Sequences were aligned using ClustalW, and a phylogenetic tree was constructed using the DNA maximum likelihood program (DNAml) and *vif* nucleotide sequences, implemented in BioEdit 7.0.9.0 (Ibis Biosciences). HIV-1 subtypes were determined using the REGA HIV subtyping tool (http://hivdb.stanford.edu/).

In addition, second-round *vif* amplicons were digested with KpnI and MscI and cloned into pCAGGS-FLAG-RRE (a FLAG-tagged Vif expression plasmid containing a Rev-responsive element), as previously described (46). A single representative clone per patient, exhibiting an amino acid sequence identical to that of the original bulk sequence, was selected for subsequent analysis. In cases where bulk sequences exhibited amino acid mixtures, either residue was permitted in the representative clone.

Virion production, APOBEC3G degradation, and viral infectivity assay. The viral infectivity assay for comparing the levels of anti-APOB EC3G activity of Vif has been previously described (46). Briefly, to prepare VSV-G-pseudotyped HIV-1 luciferase reporter viruses, 1.75×10^5 293T cells were cotransfected with 12.5 ng of pCA-hA3G-HA, 50 ng of VSV-G expression plasmid pHIT/G, 4 ng of the patient-derived Vif expression plasmid, and 0.43 μg of an empty vector (to adjust the total DNA amount to reach 1 μg) together with 500 ng of pNL-*Luc-vif*(-)*env*(-) using FuGENE 6. The amount of APOBEC3G expression plasmid was optimized to physiologically relevant levels (46). After medium exchange and DNase treatment, pseudoviruses were harvested 48 h following transfection. The p24 antigen levels in viral supernatants were measured by an HIV-1 p24-antigen capture enzyme-linked immunosorbent assay

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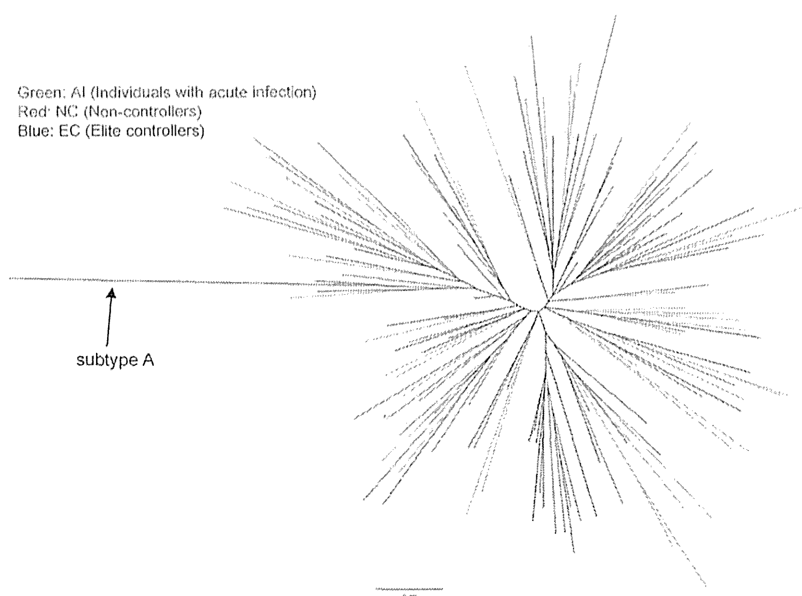

FIG 1 Phylogenetic tree of patient-derived *vif* nucleotide sequences. Maximum likelihood phylogenetic trees were computed using bulk plasma HIV RNA-derived *vif* nucleotide sequences. Green, red, and blue lines indicate AI, NC, and EC-derived sequences, respectively. All but one patient-derived *vif* were subtype B; the single subtype A sequence was excluded from all further analyses. No gross clustering was observed by patient phenotype (EC, NC, or AI).

(ZeptoMetrix Corporation). To determine the viral infectivity, 3.5×10^4 293T cells were incubated with supernatants containing 1 ng p24. After 48 h, luciferase activity was determined by the use of a ONE-Glo luciferase assay system (Promega) using a GloMax 96 Microplate Luminometer (Promega).

Levels of APOBEC3G degradation by different Vif isolates. To determine the APOBEC3G degradation efficiency of different Vif proteins, the levels of intracellular and virion-associated APOBEC3G were evaluated using representative Vif clones from 5 EC and 5 NC. A total of 7×10^5 293T cells were cotransfected with 50 ng of pCA-hA3G-HA, 200 ng of VSV-G expression plasmid pHIT/G, 16 ng of the patient-derived Vif expression plasmid, and 1.73 μg of an empty vector (to adjust the total DNA amount to reach 4 μg) together with 2 μg of pNL-*Luc-vif*(-)*env*(-) using FuGENE 6. Forty-eight hours posttransfection, pseudotyped virus was harvested and cells were lysed in 400 μl of radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor (Complete; Roche). Western blot analysis was performed using the cell lysate and purified virions. Monoclonal antibodies specific for HA (Cell Signaling Technology, Inc.), p24 (Abcam), and β-actin (Sigma-Aldrich) were used. Western blot band intensity was quantified with ImageJ 1.48 (National Institutes of Health).

Vif protein expression analysis. To confirm Vif protein expression, 293T cells were cotransfected with 200 ng of RRE-carrying FLAG-tagged patient-derived Vif expression plasmid, 200 ng of Rev expression plasmid (pCA-Rev [47]), and empty vector to reach a total of 1 μg of DNA, using the FuGENE 6 transfection reagent (Roche Applied Science). Western blot analysis was performed using extracts from the 293T cells. Antibodies specific for FLAG and β-actin were used. Note that a larger amount (200 ng) of Vif expression plasmid was used to confirm the expression of Vif proteins than was used in the viral infectivity assay (4 ng).

Sequence analysis and statistics. Statistical analysis of continuous variables was performed using the Mann-Whitney U test in GraphPad Prism 6 (GraphPad Software, Inc.). Correlations were evaluated using Spearman's rank coefficients in GraphPad Prism 6 (GraphPad Software, Inc.). Comparisons of categorical values were performed using Fisher's exact test computed in JMP Pro 9 (SAS Institute). A q value approach was employed to correct for multiple comparisons (48). In our analyses of viral genetic correlates of EC, P values of <0.05, corresponding to q values of <0.4 (indicating a 40% false-discovery rate), were considered statistically significant.

Nucleotide sequence accession numbers. The GenBank accession numbers for clonal Vif sequences are KF834928 to KF834974 (EC), KF834835 to KF834878 (AI), and KF834879 to KF834927 (NC).

RESULTS

Phylogenetic analysis of HIV-1 *vif* from patients with different disease statuses. *Vif* genes from 137 HIV-positive individuals with different disease statuses (47 EC, 46 NC, and 44 AI) were successfully amplified and sequenced. With the exception of a single EC infected with HIV-1 subtype A1 (subsequently excluded from analysis), all harbored HIV-1 subtype B. Phylogenetic analysis revealed no gross clustering of viral sequences from the different patient groups (EC, NC, and AI), indicating that the extreme viremia control exhibited by the EC patients is not explained simply by recent shared ancestry (Fig. 1). No contamination was suspected.

The anti-APOBEC3G activity of Vif proteins derived from EC was attenuated compared to that of Vif proteins from NC or AI. Patient-derived *vif* genes were cloned into FLAG-tagged mammalian expression plasmids and resequenced. For each patient, a single clone whose amino acid sequence was identical to the original bulk plasma HIV RNA sequence was selected for study. Verification of Vif protein expression in the plasmid-transfected cells was performed on a randomly selected panel of 11 EC and 10 NC *vif* genes by immunoblotting using anti-FLAG antibodies. These experiments revealed no substantial differences in Vif protein expression levels between EC-derived Vif and NC-derived Vif (Fig. 2). To assess the anti-APOBEC3G activity of patient-derived Vif proteins, VSV-pseudotyped virions harboring patient-derived Vif protein, human APOBEC3G protein, and a luciferase-reporter *vif-env*-defective HIV-1 NL4-3 DNA genome were used to infect 293T cells. Vif anti-APOBEC3G activity was measured in terms of

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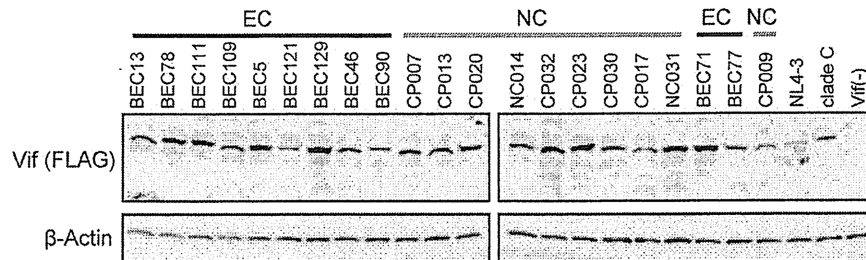

FIG 2 Protein expression of EC-derived- and NC-derived-Vif genes. To confirm Vif protein expression, Western blot analysis was performed on a randomly selected panel of 11 EC and 10 NC Vif proteins, using cellular lysates of 293T cells cotransfected with 200 ng of the RRE-carrying FLAG-tagged expression plasmids of patient-derived *vif* and pCA-Rev. These, together with the NL4-3 *vif* gene and a subtype C *vif* gene used as positive controls and Vif(-) as a negative control, are shown.

luciferase activity (represented as relative light units [RLU]) 48 h later. Overall, we observed a relatively broad range of Vif anti-AP OBEC3G activities across all patient groups studied (Fig. 3). Nevertheless, on average, Vif proteins derived from EC displayed significantly reduced activities (median $\log_{10}$ RLU, 4.54 [IQR, 4.30 to 4.66]) compared to those from NC (4.75 [4.60 to 4.92], $P < 0.0001$) and those from AI (4.74 [4.62 to 4.94], $P < 0.0001$). In contrast, no significant difference between the activities of AI- and NC-derived Vif sequences was observed.

Vif-mediated infectivity correlates inversely with cell-associated APOBEC3G levels. To examine whether differences in Vif-mediated infectivity were associated with levels of viral particle-associated or intracellular APOBEC3G, we performed Western blot analysis using lysates of purified virions and of producer cells expressing Vif proteins from 5 representative EC and 5 representative NC. When Vif proteins were coexpressed, APOBEC3G could not be detected in viral particles (data not shown). However, analysis of APOBEC3G levels in virion-producer cells revealed lower APOBEC3G levels in cells expressing NC-derived Vif than in those expressing EC-derived Vif (Fig. 4A), consistent with reduced Vif function in the latter patient group. Importantly, Vif-mediated infectivity was significantly inversely associated with cell-associated APOBEC3G levels (normalized by $Pr55^{gag}$) (Spearman's $r = -0.68$; $P = 0.035$) (Fig. 4B). Together, these data indicate that EC-derived *vif* isolates have a decreased ability to promote intracellular proteasomal degradation of APOBEC3G proteins.

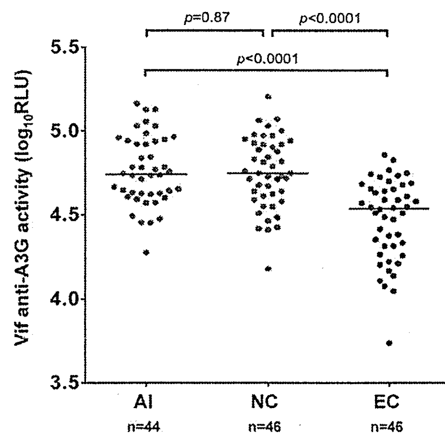

FIG 3 Comparison of anti-APOBEC3G activities of Vif proteins from AI, NC, and EC. Each dot represents the mean $\log_{10}$ RLU value corresponding to each patient, calculated from triplicate measurements. Each horizontal line represents the median $\log_{10}$ RLU of each group. The difference in $\log_{10}$ RLU values between groups was determined using the Mann-Whitney U test. RLU, relative light units; AI, individuals with acute infection; NC, noncontrollers; EC, elite controllers.

Vif functional motifs were conserved in EC. We next examined known Vif functional motifs for evidence of mutations that could explain the relative attenuation of EC-derived Vif proteins. These included the SLQYLA motif (located between codon 144 and codon 148), critical for Elongin C binding (49, 50), the HCCH motif (comprised of residues 108H, 114C, 133C, and 139H), the TQX_5ADX_2I motif (located at residues 96 to 107), critical for interaction with Cullin5 (51–54), the C-terminal PPLP motif (located between codon 161 and codon 164), required for multimerization of Vif (55–57), and the tryptophan (W) residues at positions 11, 21, 38, 79, and 89, essential for the selective suppression of APOBEC3G and APOBEC3F (58). However, with the exception of three NC who harbored polymorphisms in the SL QYLA motif, all other motifs were 100% conserved in all patients (Fig. 5). Therefore, defects in these functional motifs are unlikely to explain the elite control.

No common Vif amino acid mutations explain elite control. We next investigated the potential existence of common Vif mutations that account for elite control by comparing the frequencies of all amino acids observed in Vif sequences from EC to the frequencies of those observed in NC and AI (as a single combined group), using Fisher's exact test. At a P value of <0.05 (corresponding to $q < 0.4$), differential amino acid frequencies of the groups were observed at 4 sites (codons 30, 39, 47, and 159; total, 6 polymorphisms) (Table 1). However, at all of these sites, the consensus HIV subtype B amino acid (defined at http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html) was observed at a higher frequency in EC than in non-EC. The high prevalence of consensus amino acids at these positions in EC-derived Vif sequences indicates that their lower function (relative to NC and AI) cannot be explained by a single common substitution.

Protective HLA alleles were not associated with anti-APOBEC3G activity of Vif protein in EC. Viremia control is strongly associated with expression of particular HLA class I alleles (B*27, B*51, B*57, etc.) (2, 6, 8, 9). As such, we examined associations between HLA alleles expressed in a minimum of 5 EC or NC patients and the anti-APOBEC3G activity of their Vif proteins, using the Mann-Whitney U test (Fig. 6). AI patients were not examined, as sufficient within-host evolution might not have oc-

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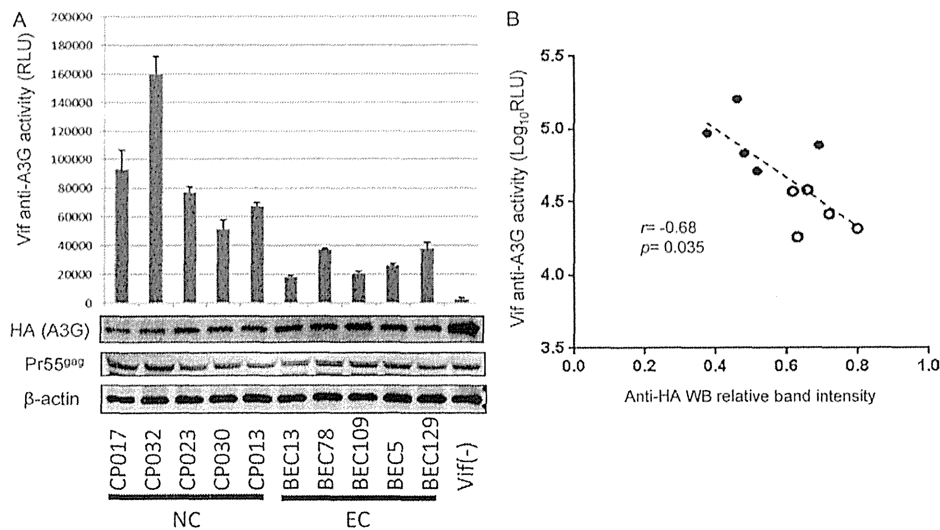

FIG 4 APOBEC3G levels in pseudovirus-producing-cell lysate and Vif anti-APOBEC3G activity. (A) For a randomly selected panel of 5 NC and 5 EC *vif* genes, Vif anti-APOBEC3G activity (bar graph), APOBEC3G protein levels (top panel), $Pr55^{gag}$ levels (middle panel), and β-actin levels (bottom panel) within pseudovirus-producing cells were evaluated using immunoblotting and cellular lysates of 293T cells cotransfected with 50 ng of pCA-hA3G-HA, 200 ng of the VSV-G expression plasmid pHIT/G, 16 ng of the patient-derived Vif expression plasmid, and 1.73 μg of an empty vector together with 2 μg of pNL-Luc-vif (-)env(-) using FuGENE 6. These, together with Vif(-) as a control, are shown. Error bars of the Vif anti-APOBEC3G activity (RLU) (bar graph) represent standard deviations calculated from triplicate infection assays. (B) Correlation between Vif anti-APOBEC3G activity (Log_{10} RLU) and APOBEC3G protein levels (adjusted by $Pr55^{gag}$ intensity) within the pseudovirus-producing cells shown in panel A. Closed symbols and open symbols represent NC and EC, respectively. A significant inverse correlation is observed. A dotted line is added to help visualize the trend. WB, Western blot.

curred at this early stage for host HLA effects to be detected. No significant differences in Vif function were observed between EC or NC patients harboring protective HLA alleles (B*27, B*51, B*57) and those lacking these alleles. Of note, higher Vif function was associated with HLA-A*03 in EC ($P = 0.007$, $q = 0.30$). Together, these results suggest that immune pressures mediated by canonical "protective" HLA class I alleles are unlikely to be playing a major role in the reduced Vif activity in EC, though the modest statistical power of our data set must be noted.

DISCUSSION

In this study, we found that the average anti-APOBEC3G activity of Vif proteins derived from elite controllers was significantly lower than that of Vif proteins derived from noncontrollers and individuals with acute HIV infection, despite comparable levels of Vif protein expression in the controller and noncontroller patient groups. However, this functional reduction was unlikely to be explained by a common single Vif mutation among EC and did not correlate with the HLA class I alleles expressed by the host.

Previous studies evaluating EC-derived viruses demonstrated that the *in vitro* replication capacity associated with Gag (20) or Pol (21), the entry efficiency of Env (18), and the function of Nef from EC (19) were attenuated compared to those from NC. As within-host HIV genetic diversity (and *in vitro* replicative fitness) tends to increase during the natural course of HIV-1 infection (59, 60), at a rate that is dependent on within-host viral replication, it is possible that the higher level of intrahost viral evolution in NC (who have high viral loads) than in EC (who have undetectable viral loads) could serve as a confounder. Specifically, if transmitted viruses are generally less fit than those isolated later in infection (61), we reasoned that it was necessary to also compare the function of EC-derived viral sequences to the function of those derived from acutely infected individuals. If they were comparable, this would suggest that the lower function of EC-derived sequences was likely due to limited within-host evolution over the disease course. But if the function of EC-derived *vif* sequences was lower than that of AI-derived *vif* sequences, then this would be compatible with acquisition of unusually attenuated viral strains and/or with selection of unusual function-reducing mutations over the disease course. It is for this reason that our study included AI as an additional comparison group.

Importantly, we demonstrated that the anti-APOBEC3G activities of Vif proteins in EC were significantly lower than those of Vif proteins in noncontrollers, regardless of whether the latter patients were assessed in acute infection or chronic infection. This indicates that reduced Vif function in EC does not simply reflect reduced intrahost viral evolution in these patients. Though the cross-sectional nature of our study does not allow us to discern whether this attenuated Vif function is a cause or a consequence of viremia control and the clinical implications remain to be determined, our results nevertheless suggest that transmitted/founder viruses acquired by EC are already unusually attenuated compared to those acquired by individuals who subsequently progress clinically and/or that Vif function in EC is further attenuated over the infection course as a result of adaptation to host-driven pressures. It is also important that, while Vif function in EC was on average significantly lower than that in noncontrollers, there was substantial functional overlap between the two groups. In a previous study using the same assay (46), a 0.5 $\log_{10}$ RLU decrease in anti-APOBEC3G activity resulted in a significant increase in G-to-A hypermutation frequency, suggesting that an *in vitro* functional reduction of this magnitude could be biologically relevant. Eleven (24%) of our 46 EC-derived *vif* isolates exhibited anti-AP

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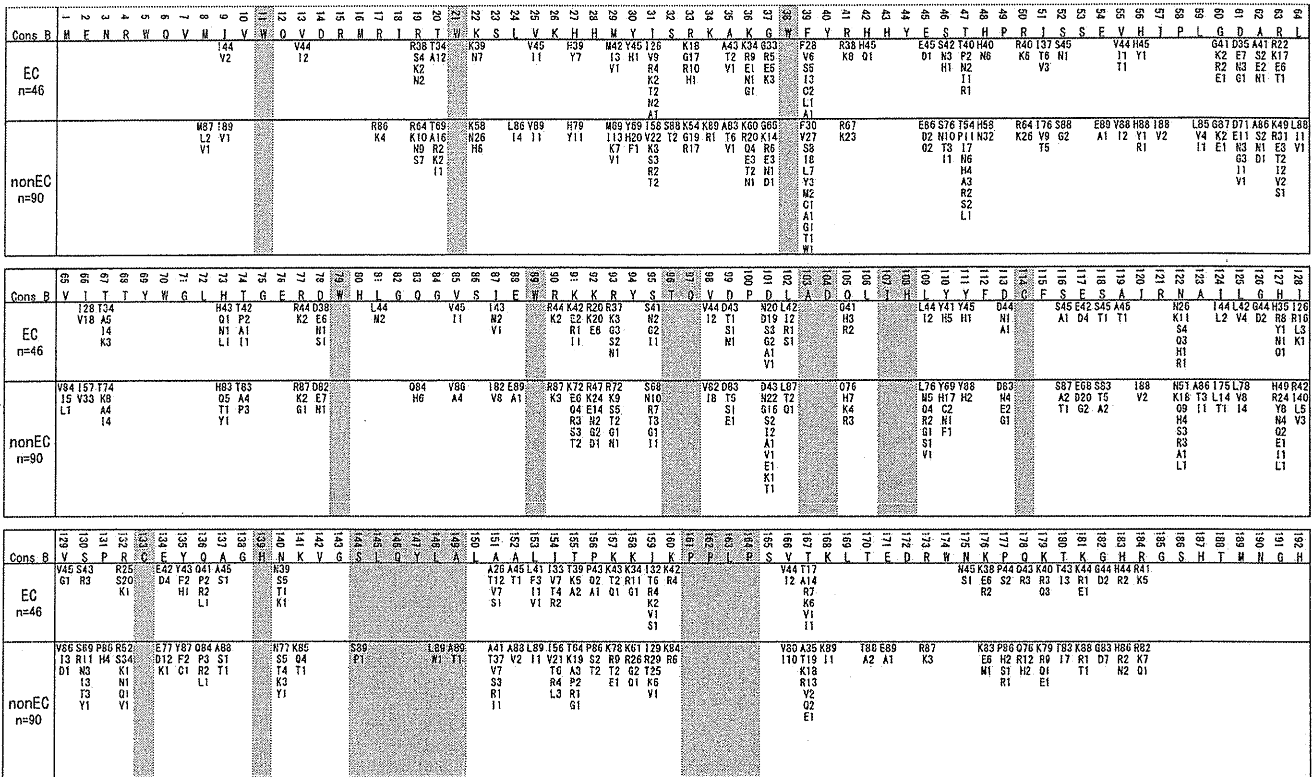

FIG 5 Vif amino acid frequencies in EC and non-EC. The top row of capital letters represents the HIV-1 Vif subtype B consensus (Cons B) amino acid sequence. For all codons that were less than 100% conserved in our data set, the frequencies of observed amino acids in EC and non-EC are shown below the consensus data. Known conserved Vif functional motifs are highlighted in gray. Codon positions 1 to 7 and 185 to 192 were the second PCR primer sites.

OBEC3G activities that were at least $-0.5 \log_{10}$ RLU lower than the median anti-APOBEC3G activity of NC, indicating that the *in vitro* reductions observed in this study were of a magnitude that could be biologically relevant. In order to investigate the mechanisms by which Vif function is attenuated in EC, Vif amino acid sequences were analyzed. However, no major mutations were observed within known functional motifs, and no common single polymorphisms were observed that could explain EC status. We speculate that the relative Vif defects observed in EC may attributable to combinations of minor polymorphisms, though further investigations using higher-powered data sets will be necessary to confirm this.

Among EC, certain HLA class I alleles are overrepresented, in particular, HLA-B*57, B*27, and B*51, suggesting that HIV-1-specific cytotoxic T-lymphocyte (CTL) responses restricted by these alleles are crucial for viremia control (9, 10, 12). CTL escape mutations restricted by these protective HLA alleles can also reduce viral replication capacity (20, 62–68). Accordingly, the *in vitro* replication capacities of recombinant viruses expressing Gag and Pol, as well as the protein function of Nef sequences, have been reported to be attenuated in EC, especially in HLA-B57-positive (HLA-B57^{+}) individuals (19–21). In contrast, in the present study, no association was found between attenuated Vif function and HLA class I alleles, including HLA-B*57, though the relatively modest statistical power of the present data set is noted. As reported previously, the magnitude and breadth of the HIV-specific CTL responses targeting accessory/regulatory proteins (Tat, Rev, Vpr, Vif, and Vpu) were lower than those of the HIV-

TABLE 1 Vif polymorphisms associated with patient phenotype (EC versus non-EC)[a]

Amino acid residue	No. (%) of EC with indicated polymorphism	No. (%) of EC without indicated polymorphism	No. (%) of non-EC with indicated polymorphism	No. (%) of non-EC without indicated polymorphism	*P* value	*q* value	Subtype B consensus
030H	1 (2)	45 (98)	20 (22)	70 (75)	0.0019	0.21	Y
030Y	45 (98)	1 (2)	69 (74)	21 (23)	0.0010	0.21	Y
039F	28 (61)	18 (39)	30 (32)	60 (65)	0.0032	0.24	F
047T	40 (87)	6 (13)	54 (58)	36 (39)	0.0015	0.21	T
159I	32 (70)	14 (30)	29 (31)	61 (66)	<0.0001	0.0045	I
159R	4 (9)	42 (91)	29 (31)	61 (66)	0.0027	0.24	I

[a] All polymorphisms, regardless of their observed frequency in our data set, were analyzed. Polymorphisms with a *P* value of <0.05 and *q* value of <0.4 are reported. EC, elite controller.

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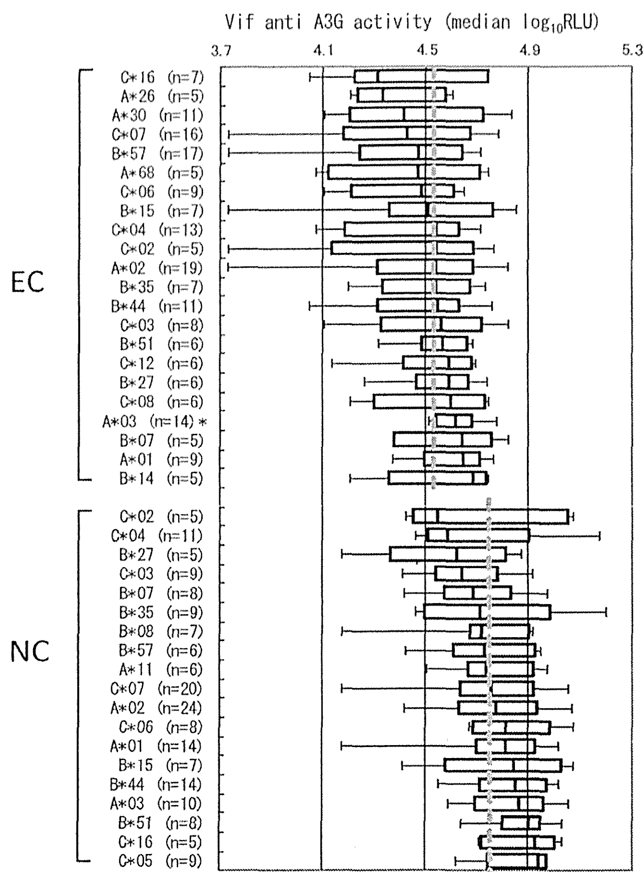

FIG 6 Association between HLA class I allele expression and Vif activity. Box and whisker plots depict the median (vertical line), interquartile range (box), and range (whiskers) of log_{10} RLU (Vif activity), stratified by the HLA class I alleles expressed by the patient. Analysis was limited to the HLA class I alleles observed in a minimum of 5 individuals. The dotted vertical line indicates the median Vif activity for each patient group. The asterisk next to A*03 (EC) indicates that EC expressing A*03 exhibited significantly higher Vif activities than those not expressing A*03 (Mann-Whitney U-test; $P = 0.007$, $q = 0.30$). All other comparisons were non-statistically significant at a P of <0.05 and a q of <0.4.

specific CTL responses targeting Gag, Pol, and Nef in EC, viremic controllers, and chronic progressors (10, 11). Therefore, the discordance with the findings in the previous studies on other HIV proteins may be explained by lower levels of CTL responses to the Vif protein.

The present study featured several limitations. First, as a marker for anti-APOBEC3G activity of Vif *in vitro*, we analyzed Vif-mediated infectivity that is mediated by multiple mechanisms, including promotion of the degradation of APOBEC3G (24–26, 31, 32), prevention of encapsidation of APOBEC3G (69–71), and interaction with other APOBEC3G proteins (APOBEC3DE/3F/3H) (72–74). We failed to evaluate the levels of virion encapsidation of APOBEC3G, since encapsidated APOBEC3G was not detected in the presence of Vif protein under our physiologically optimized *in vitro* conditions. At the least, we found that Vif-induced intracellular degradation of APOBEC3G could be one of the possible mechanisms of attenuated Vif function in EC. Although we did not evaluate Vif's interactions with other APOBEC3G proteins, the intrinsic potencies of other APOBEC3 proteins (such as APOBEC3DE/3F/3H) appear to be lower than those of APOBEC3G (46, 72–75). Moreover, we did not observe interpatient differences in Vif-mediated infectivity in control experiments where the APOBEC3G expression plasmid was not delivered into 293 T cells (data not shown). As such, our method properly evaluates Vif's *in vitro* activity against its principal target, APOBEC3G.

Second, differences in sampling dates could confound Vif functional assessments in two ways: via intrapatient viral evolution over the infection course and/or via interpatient viral evolution over the epidemic's course. The AI patient group was included in the study to indirectly address the first confounder (as intrapatient evolution is minimized in AI due to the limited elapsed time since infection). With respect to the latter confounder, we observed no correlation between calendar date of sampling and Vif-mediated infectivity in each patient group (Spearman's $r = -0.19$ in EC, $r = -0.09$ in NC, and $r = 0.06$ in AI; all $P > 0.05$). This suggests that our results are not substantially confounded by the population-level evolution of HIV-1 over the epidemic's course, though further study is warranted to further investigate the evolution of Vif sequence and function at a population level. Third, only a single Vif clone from each subject was studied (though we took care to select a clone identical to the bulk plasma viral sequence in each case). Given the large genetic diversity in NC, a potential bias could not be ruled out, especially in cases where the bulk sequence featured amino acid mixtures (only one of which was represented in the selected clone). To further address this, we repeated the Vif functional assessments using a randomly selected clone, which yielded results consistent with the primary analysis (data not shown). Therefore, we feel that our observed results are unlikely due to selection biases associated with selection of a single clone per patient.

Fourth, our use of an *in vitro* assay to evaluate the anti-APOBEC3G function of patient-derived HIV Vif proteins does not allow us to investigate host-related factors such as APOBEC3G polymorphisms and cellular expression levels. Unfortunately, assessment of APOBEC3G levels or polymorphisms was not possible, as peripheral blood mononuclear cells (PBMCs) were not available for the studied patients. A previous report indicated that *in vivo* APOBEC3G mRNA levels correlated inversely with the level of viremia (76), although the data from another report are conflicting (77). Likewise, attempts to identify SNPs within APOBEC3G associated with HIV disease progression have not yielded any significant hits (78). As such, the potential impact of host factors should be addressed in future studies as well.

In conclusion, we observed that the average anti-APOBEC3G activity of Vif derived from elite controllers was significantly reduced compared to the activity of those derived from noncontrollers, regardless of the infection stage. This reduced function was unlikely to be explained by common Vif mutations in EC; as such, further study will be required to fully elucidate the genotypic mechanisms underlying the attenuated Vif function in EC. Nevertheless, these results extend the growing body of evidence supporting the idea of relative attenuation of viral protein function in EC-derived HIV sequences.

ACKNOWLEDGMENTS

This study was supported in part by The International HIV Controllers Study (IHCS), funded by the Bill and Melinda Gates Foundation (F.P. and B.D.W.),

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the AIDS Healthcare Foundation (F.P.), and the Harvard University Center for AIDS Research (CFAR), an NIH-funded Center for AIDS Research (P30 AI060354), which is supported by the following NIH cofunding and participating institutes and centers: NIAID, NCI, NICHD, NHLBI, NIDA, NIMH, NIA, FIC, and OAR. Members of IHCS can be found at http://ragoninstitute.org/hivcontrollers/health-professionals/study-members/the-international-hiv-controllers-study-members/. Z.L.B. is the recipient of a New Investigator Award from the Canadian Institutes of Health Research (CIHR) and a scholar award from the Michael Smith Foundation for Health Research (MSFHR). This work was supported in part by a contract research fund from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for the Program of Japan Initiative for Global Research Network on Infectious Diseases (10005010) (A.I.); the Global COE Program (Center of Education and Research for Advanced Genome-Based Medicine—for personalized medicine and the control of worldwide infectious diseases) of MEXT (F06) (A.I.); Japan Society for the Promotion of Science KAKENHI (22390203) (T.M.); JSPS KAKENHI (22590428) (K.T.); JSPS KAKENHI (25293226) (A.K.-T.); JSPS KAKENHI (24790437) (N.H.); the Ministry of Health, Labor, and Welfare of Japan (Research on HIV/AIDS project no. H24-005, H24-008, and H25-010) (K.T.); Grants for AIDS Research from MHLW of Japan (H24-AIDS-IPPAN-008) (A.K.-T.); Grants for AIDS Research from MHLW of Japan (H25—AIDS-IPPAN-006) (N.H.); and Research on International Cooperation in Medical Science, Research on Global Health Issues, Health and Labor Science Research Grants, MHLW of Japan (H25-KOKUI-SITEI-001) (A.I.).

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