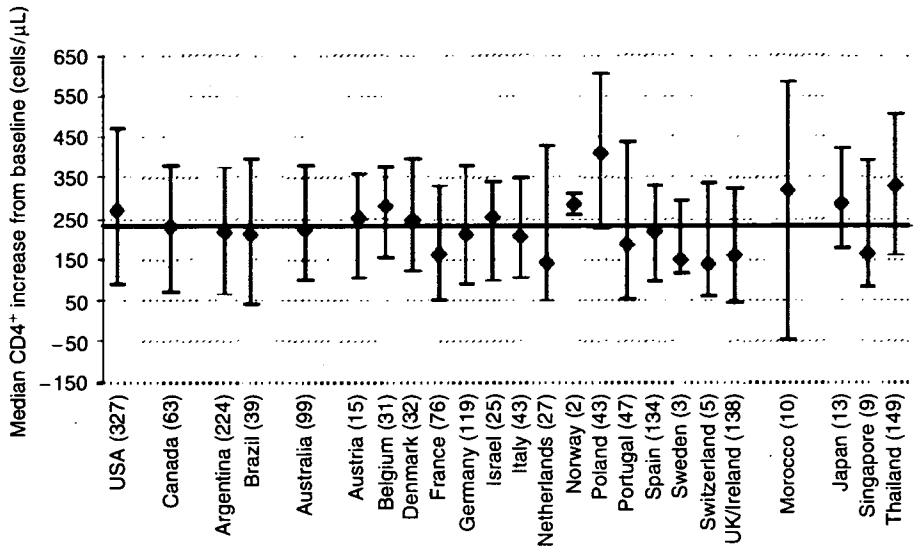

Fig. 2 The median (with interquartile range) change in CD4 cell count from baseline according to country (n) compared with the median change overall (thick horizontal line).

Table 2 Regression analysis to investigate predictors of the change in CD4 cell count between baseline and 8 months for the 1673 patients

	Single variable analysis		Multivariable analysis[a]		Multivariable analysis[b]	
Variable	Difference in mean CD4 cell count (95% CI)	*P*-value	Difference in mean CD4 cell count (95% CI)	*P*-value	Difference in mean CD4 cell count (95% CI)	*P*-value
CD4 nadir (per 100 cells/μL)	54.6 (45.2, 64.0)	< 0.0001	29.8 (18.7, 40.8)	< 0.0001	46.2 (35.5, 57.0)	< 0.0001
Last CD4 cell count prior to baseline (per 100 cells/μL)	48.0 (40.0, 55.9)	< 0.0001	36.0 (27.3, 44.7)	< 0.0001	-	-
CD4 cell count at baseline (per 100 cells/μL)	26.2 (17.9, 34.6)	< 0.0001	-	-	9.8 (0.9, 18.7)	0.03
Age at baseline (per 5 years older)	−21.5 (−29.6, −13.5)	<0.0001	−11.7 (−19.8, −3.5)	0.005	−10.5 (−18.7, −2.4)	0.011
Time between the start of ART and baseline (per year longer)	−15.5 (−20.1, −11.0)	<0.0001	−8.3 (−13.1, −3.6)	0.001	−7.3 (−12.0, −2.6)	0.002
Total rIL-2 dose received during first cycle (per 15 MIU)	74.5 (52.5, 96.5)	< 0.0001	73.1 (51.7, 94.4)	< 0.0001	72.6 (51.3, 94.0)	< 0.0001
Baseline HIV-RNA (<500/≥500 copies/mL)	49.1 (14.1, 84.1)	0.006	56.1 (21.5, 90.8)	0.002	72.5 (38.4, 106.6)	< 0.0001
BMI (per 5 units)	−24.6 (−43.8, −5.5)	0.012	−22.8 (−41.7, −3.9)	0.018	−20.7 (−39.7, −1.8)	0.032
Race		< 0.0001		0.003		0.002
White	Ref.		Ref.		Ref.	
Black	94.0 (43.9, 144.1)		79.7 (31.5, 127.9)		83.4 (35.3, 131.5)	
Asian	114.4 (68.8, 160.0)		48.3 (−0.5, 97.1)		40.6 (−5.4, 86.6)	
Other	−5.9 (−82.0, 70.2)		−13.9 (−87.0, 59.2)		−23.1 (−96.1, 49.9)	

[a]Analysis including the last CD4 cell count before baseline in the model.
[b]Analysis including the baseline CD4 cell count in the model.
ART, antiretroviral therapy; BMI, body mass index; CI, confidence interval; rIL-2, recombinant interleukin-2.

CD4 cell count response

After 8 months of follow up, the median (IQR) CD4 cell count was 703 (531, 960) cells/μL with a median (IQR) increase of 233 (90, 411) cells/μL from baseline to 8 months. There were significant variations in the change from baseline according to the country of origin (Fig. 2): the median increase in CD4 cell counts ranged from 137 to 411 cells/μL among the 24 countries (UK and Ireland are combined). To account for this variation we included region in a multivariable model.

The most significant predictors of response to rIL-2 in multivariable analysis ($R^2 = 0.17$) were CD4 nadir, last CD4 cell count prior to baseline, time on ARTs prior to baseline, baseline HIV-RNA level, age and race in addition to the total dose of rIL-2 received during the first cycle (Table 2a). Assumptions of a linear relationship between the continuous variables and CD4 cell count change seemed reasonable after visual inspection of normal plots. After adjustments for covariates for which there was a statistically significant association, there was a mean [95% confidence interval (CI)] 11.7 (3.5, 19.8) cells/μL smaller increase in CD4 cell count for every 5 years older at baseline, and patients with a suppressed baseline HIV-RNA had a mean (95% CI) 56.1 (21.5, 90.8) cells/μL greater increase in CD4 cell count compared with those who did not.

Patients with a good immunological status at baseline, as determined by a high CD4 cell count prior to baseline, did better, with an estimated mean (95% CI) increase of 36.0 (27.3, 44.7) cells/μL per 100 cells/μL higher last CD4 cell count, and every 100 cells/μL higher nadir CD4 was associated with an overall mean (95% CI) increase of 29.8 (18.7, 40.8) cells/μL. Both the nadir CD4 count and the last CD4 count prior to entry were split into quartiles and the change in CD4 cell count from baseline was investigated. There were significant increases in the change in CD4 cell count from baseline with increasing CD4 nadir quartile ($P<0.0001$) and with increasing latest CD4 cell count quartile ($P<0.0001$). Patients whose CD4 nadir was in the lowest quartile (≤ 84 cells/μL) and whose latest CD4 cell count prior to entry was ≤ 350 cells/μL only had a mean increase of 144.8 cells/μL between baseline and month 8. If they had a higher CD4 cell count just prior to baseline then their rIL-2-induced increases were greater (Table 3).

A linear relationship existed between total rIL-2 dose administered in the first cycle and the change in CD4 cell count: patients who received the most rIL-2 in the first cycle showed the best immunological responses over 8 months (mean 73.1 cells/μL greater increase per 15 MIU higher). Patients receiving > 60 MIU during their first cycle demonstrated the greatest response, with a mean (95% CI) 268.8 (85.2, 452.3) cells/μL higher increase in CD4 cell count compared with patients who received ≤ 15 MIU, after adjustments in a multivariable analysis (Fig. 3). With the inclusion of baseline CD4 cell count instead of last CD4

Table 3 Mean increase in CD4 cell count according to CD4 nadir and last CD4 cell count prior to entry

	Last CD4 cell count prior to entry (cells/μL)				
CD4 nadir (cells/μL)	**≤ 350**	**351–446**	**447–573**	**> 573**	**Total**
84	144.8 ($n = 151$)	173.4 ($n = 126$)	238.0 ($n = 93$)	226.2 ($n = 29$)	194.4 ($n = 419$)
85–190	196.3 ($n = 127$)	211.8 ($n = 121$)	246.1 ($n = 95$)	280.0 ($n = 56$)	234.0 ($n = 421$)
191–293	257.6 ($n = 88$)	304.0 ($n = 98$)	320.4 ($n = 107$)	388.8 ($n = 108$)	299.0 ($n = 416$)
> 293	217.9 ($n = 44$)	292.5 ($n = 60$)	397.6 ($n = 108$)	464.6 ($n = 185$)	395.7 ($n = 417$)
Total	181.5 ($n = 399$)	224.6 ($n = 399$)	321.0 ($n = 401$)	393.0 ($n = 397$)	279.9 ($n = 1596$)[a]

[a]Seventy-seven patients did not have both a CD4 nadir and a CD4 cell count recorded prior to baseline.

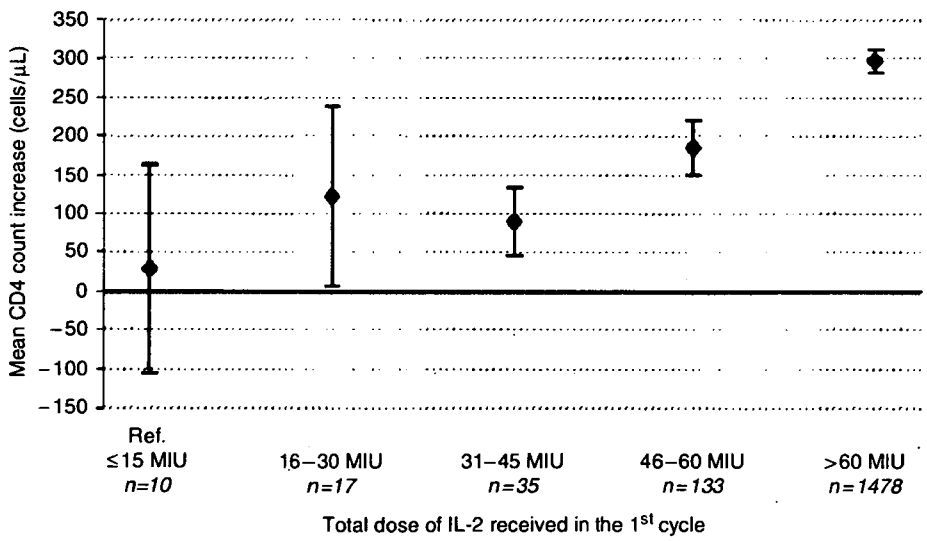

Fig. 3 The mean (with 95% confidence interval) increase in CD4 cell count between baseline and 8 months of follow up according to the total dose of recombinant interleukin-2 (rIL-2) received in the first cycle.

cell count prior to entry, the association with the predictors studied did not change appreciably ($R^2 = 0.14$; Table 2b).

In single variable analysis, HBV/HCV coinfection was not predictive of immunological response to rIL-2 [a mean (95% CI) 22.1 (−42.6, 86.8) cells/μL smaller increase for HBV-coinfected individuals and a mean (95% CI) 12.7 (−30.3, 55.7) cells/μL smaller increase for HCV-coinfected individuals]. In addition, HBV/HCV coinfection had no influence on the estimates of other covariates in a multivariable analysis. There was no relationship noted between gender and CD4 cell count response [mean (95% CI) 3.1 (−34.1, 40.2) cells/μL greater increase for female patients] in either single or multivariable analysis.

Discussion

This is the largest existing study in which it is possible to investigate the relationship between rIL-2 and CD4 cell count recovery. We evaluated immunological response for 1673 HIV-1-positive patients who were receiving cART and rIL-2 and related the change in CD4 cell count between baseline and 8 months to baseline characteristics. We observed a median (IQR) increase of 233 (90, 411) cells/μL in 8 months, with a median (IQR) 8-month CD4 cell count of 703 (531, 960) cells/μL. This rise is lower than that found in some studies that have investigated the effect of IL-2 on CD4 cell count over a longer period of time, administered through different routes or in patients who were ART-naïve prior to staring IL-2 [11,17–19]. This could reflect poorer adherence in ESPRIT patients; however, the increases found here are similar to those found in other studies [20,21].

Previous studies have shown a strong relationship between IL-2-induced CD4 cell count and baseline CD4 cell count [11,12]. We decided, *a priori*, to use the last CD4 cell count prior to baseline to classify patients at entry because baseline CD4 cell counts are used to calculate the outcome variable (CD4 cell count change from this baseline), and therefore a spurious association between CD4 response and baseline CD4 cell count would be likely to occur. The correlations between the last CD4 cell count before baseline and baseline CD4 count [median (IQR) of 91 (56, 132) days between measurements; Spearman's rank correlation coefficient = 0.71] and between the last CD4 cell count before baseline and the 8-month CD4 cell count [median of 330 (293, 372) days between measurements; Spearman's rank correlation coefficient = 0.58] are similar, and therefore the effect of regression to the mean can be substantially reduced by classifying patients on their last CD4 cell count before baseline and relating it to rIL-2-induced changes from the baseline CD4 cell count [15]. In the absence of rIL-2, these correlations may have been even closer. Inclusion of baseline CD4 cell count in the model instead of the last CD4 cell count before entry did not change the other covariates substantially; the same covariates were identified, all with similar estimates to those in the initial model.

Although many factors that influence CD4 cell count recovery on rIL-2 when combined with cART (i.e. baseline CD4 cell count, nadir CD4 cell count, baseline HIV-RNA and age) are related to immunological recovery from cART alone, patients receiving cART without rIL-2 experienced smaller CD4 cell count increases. In the year prior to baseline, patients with a nadir CD4 cell count of 190 (84, 293) cells/μL experienced a median (IQR) increase in CD4 cell count of 30 (−122, 199) cells/μL after receiving cART alone for a median (IQR) duration of 4 (2, 6) years, whereas on receipt of rIL-2 the same patients experienced a median (IQR) increase of 233 (90, 411) cells/μL after only 8 months. We wanted to see whether the same predictors of response could be identified in patients receiving rIL-2 compared with the established predictors of immunological response in patients on cART not receiving rIL-2 [22,23].

The greater CD4 cell count increases in black compared with white patients were consistent across clinical sites (data not shown). In 14 (82%) of 17 sites where there were sufficient numbers of black and white patients (> 10% in each category), greater CD4 cell count increases were seen amongst black patients, but because of the limited number of patients at each site, only at three sites did this reach statistical significance (i.e. $P < 0.10$). In three sites, greater CD4 cell count increases were noted amongst white patients, but none of these differences was significant. The greater increase in CD4 cell count identified in black patients may be attributable to genetic factors yet to be identified. We shall have to wait for completion of ESPRIT to see whether these additional CD4 cells are associated with clinical benefit. Most other studies looking at IL-2-induced increases have examined a more homogeneous population with a limited number of nonwhite patients, and therefore a proper examination of racial differences may not have been possible [9,11,20,21,24,25].

ESPRIT is a multicentre study and consequently different practices on dose reduction and management of rIL-2 toxicities may have developed. Although we have tried to harmonize this by adjusting our models for the region of origin, there may be other factors that we have not been able to take into account. The geographical variation seen in our study is likely to reflect racial and treatment management differences.

It is important to identify patients who will respond to lower, less toxic doses of rIL-2, because these patients are more likely to stay on treatment and continue cycling. However, we excluded all Vanguard patients starting at doses < 7.5 MIU bid (1.5 or 4.5 MIU) because the CD4 cell

count response for these patients has already been reported. We chose a dose of 7.5 MIU for ESPRIT because this dose resulted in large CD4 cell count increases in other studies, including the Vanguard studies. Our protocol permitted dose reductions in 1.5-MIU increments in the event of toxicities. Based on earlier studies, doses of 6.0 and 4.5 MIU also led to good CD4 cell count responses. By focusing on the group of patients who started rIL-2 with the 7.5 MIU dose, the rIL-2 dosing strategy in ESPRIT could be more clearly assessed in terms of adherence, CD4 cell count response and baseline predictors.

Most studies investigating response to IL-2 have highlighted immunological improvements, even at low IL-2 doses [9,17,25]. Although we have shown a significant relationship between higher levels of rIL-2 during each cycle and CD4 cell count improvements, the dose of rIL-2 should be reduced if toxicity continues to be a problem, so that rIL-2 can still be used to compliment ART. We still need to relate the effects of rIL-2 to the host immunophenotype and/or viral phenotype (e.g. chemokine receptor polymorphisms or Syncytium Inducing/Non-Syncytium Inducing (SI/NSI) phenotype) to see if there are underlying biological factors that will predict how successful treatment with IL-2 will be.

There are several limitations to this study. We investigated response to IL-2 after the ESPRIT induction phase; this was only 8 months long, which is a relatively short period to examine CD4 cell count recovery. It would be interesting to see whether the same predictors of response are identified after a longer duration of follow up. Another limitation was that this study only investigated the impact of rIL-2 on the total CD4 cell count. Data on CD4 cell percentage and on naïve and memory T-cell subsets would help us understand the potential for these induced CD4 cells to have a clinical benefit.

In conclusion, rIL-2 substantially increased CD4 cell counts in a diverse population of patients with baseline CD4 cell counts ≥ 300 cells/μL in this study. The main aim of treatment for individuals with HIV infection is to prolong survival, and improving the immune system with rIL-2 may help to reduce clinical progression. We will evaluate whether rIL-2 can be used in combination with cART to reduce clinical progression when follow up is completed in the two clinical trials: ESPRIT and Study of IL-2 in People With Low CD4 Counts on Active Anti-HIV Therapy (SILCAAT) (a trial similar to ESPRIT but in a more immunocompromised population).

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Successful Efavirenz Dose Reduction in HIV Type 1–Infected Individuals with Cytochrome P450 2B6 *6 and *26

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Background. Efavirenz (EFV) is metabolized primarily by cytochrome P450 2B6 (CYP2B6), and high plasma concentrations of the drug are associated with a G→T polymorphism at position 516 (516G→T) of *CYP2B6* and frequent central nervous system (CNS)–related side effects. Here, we tested the feasibility of genotype-based dose reduction of EFV.

Methods. *CYP2B6* genotypes were determined in 456 human immunodeficiency virus type 1 (HIV-1)–infected patients who were receiving EFV treatment or were scheduled to receive EFV-containing treatment. EFV dose was reduced in *CYP2B6* 516G→T carriers who had high plasma EFV concentrations while receiving the standard dosage (600 mg). EFV-naive homozygous *CYP2B6* 516G→T carriers were treated with low-dose EFV. In both groups, the dose was further reduced when plasma EFV concentration remained high.

Results. *CYP2B6* 516G→T was identified in the **6* allele (found in 17.9% of our subjects) and a novel allele, **26* (found in 1.3% of our patients). All EFV-treated *CYP2B6* **6/*6* and **6/*26* carriers had extremely high plasma EFV concentrations (>6000 ng/mL) while receiving the standard dosage. EFV dose was reduced to 400 mg for 11 patients and to 200 mg for 7 patients with persistently suppressed HIV-1 loads. EFV-containing treatment was initiated at 400 mg in 4 *CYP2B6* **6/*6* carriers and one **6/*26* carrier. Two of them still had a high plasma EFV concentration while receiving that dose, and the dose was further reduced to 200 mg, with successful HIV-1 suppression. CNS-related symptoms improved with dose reduction in 10 of the 14 patients, although some had not been aware of the symptoms at initial dosage.

Conclusions. Genotype-based EFV dose reduction is feasible in *CYP2B6* **6/*6* and **6/*26* carriers, which can reduce EFV-associated CNS symptoms.

Efavirenz (EFV) is an important anti–HIV-1 agent in current combination treatment and is usually prescribed at a fixed dosage of 600 mg once daily [1, 2]. The plasma concentration of EFV varies widely in individuals, and the prevalence of CNS symptoms is higher in those with high concentrations [3]. EFV is metabolized mainly by cytochrome P450 2B6 (CYP2B6), and its concentration was reported to be associated with the *CYP2B6* 516G→T genetic polymorphism [4–8]. Previously, we reported that all Japanese patients with the 516TT genotype had extremely high EFV concentrations (>6000 ng/mL), without exception [4]. However, other studies reported some exceptional cases of subjects with the 516TT genotype with normal concentrations, although most of the

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516TT carriers had high concentrations [5–8]. The difference between our data and those of others may reflect polymorphisms other than 516G→T in *CYP2B6*. If this is the case, analysis of other polymorphisms and determination of the *CYP2B6* haplotype may be helpful in predicting EFV plasma levels. In the present study, we determined the *CYP2B6* haplotype of 456 HIV-1–infected patients and analyzed its relationship with EFV concentration in 111 of them. Furthermore, we reduced the EFV dose in 12 patients whose EFV concentrations had been high while receiving the standard dosage. We also used reduced doses of EFV in 5 EFV-naive patients in whom EFV concentration was predicted to become extremely high while receiving the standard dosage, on the basis of *CYP2B6* haplotype determination.

SUBJECTS, MATERIALS, AND METHODS

Patients. This analysis included 60 previously reported HIV-1–infected individuals at the International Medical Center of Japan (IMCJ) [4] and another group of 396 HIV-1–infected patients who were receiving treatment of the standard dosage (600 mg once daily) of EFV or were scheduled to begin receiving EFV-containing treatment at the following 11 hospitals in Japan: Hokkaido University (Sapporo), Sendai Medical Center (Sendai), Niigata University (Niigata), Higashi Saitama Hospital (Hasuda), IMCJ (Tokyo), Ishikawa Prefecture Central Hospital (Kanazawa), Nagoya Medical Center (Nagoya), Osaka National Hospital (Osaka), Hiroshima University (Hiroshima), Kyushu Medical Center (Fukuoka), and Kumamoto University (Kumamoto). The ethics committee of each hospital approved this study, and each participant gave written informed consent.

CYP2B6 *genotype.* DNA samples were extracted from peripheral blood specimens obtained from participants, and genotyping of *CYP2B6* 64C→T (*rs8192709*), 415A→G (*rs12721655*), 499C→G (*rs3826711*), 516G→T (*rs3745274*), 777C→A (*rs* number not available), 785A→G (*rs2279343*), 1375A→G (*rs* number not available), and 1459C→T (*rs3211371*) was performed by allele-specific fluorogenic 5′ nuclease chain reaction assay with predesigned primers and TaqMan MGB probes (TaqMan SNP Genotyping Assay; Applied Biosystems) or previously published primers and MGB probes [4]. In subjects confirmed to carry 499C→G, all 9 exons of the *CYP2B6* gene were amplified with previously published primers [9], and their DNA sequences were directly determined. For haplotype analysis of the *CYP2B6* allele, PCR amplification of the genomic region (3130 bp) containing exons 4 and 5 was performed using sense primer 5′-AACTGTACTCACTCCCAGAGT-3′ and antisense primer 5′-CTCCCTCTGTCTTTCATTCTGT-3′. The amplified PCR product was subjected to subcloning, and the DNA sequence of each clone was determined. For genotyping of *CYP2B6* 983T→C (*rs28399499*), new primers and probes were designed as follows: forward primer, 5′-GCCTGAAATGCCTCTTTAAAATGAGATTC-3′; reverse primer, 5′-GCGATGTGGGCCAATCAC-3′; VIC probe for 983T, 5′-CTGTTCAATCTCCC-3′; and FAM probe for 983C, 5′-CTGTTCAGTCTCCC-3′. The obtained genotyping results of *CYP2B6* 983T→C for >10 patients were confirmed by direct sequencing of exons 7 and 8 with use of primers published elsewhere [9].

Plasma EFV concentration. Samples of peripheral blood were collected during a daytime office visit (9–16 h after the patient took EFV) from the patients who had received EFV treatment at 600-mg dose at bedtime for >4 weeks. EFV concentration was measured by the reverse-phase high-performance liquid chromatography (HPLC) method [10]. For cases of EFV-dose reduction, plasma concentration was measured >2 weeks after the change in EFV dose. Differences in EFV concentrations between groups were examined for statistical significance with Student's *t* test. A P value <.05 denoted the presence of a statistically significant difference.

RESULTS

Novel* CYP2B6 *allele. The *CYP2B6* genotype was analyzed in 456 HIV-1–infected patients, including 442 Japanese, 8 other Asians, and 6 others. During the analysis, we noticed that some patients had the *CYP2B6* 499C→G polymorphism, substituting Ala for Pro at the 167th amino acid, which is already registered in the SNP Database, although the *CYP2B6* allele containing 499G had not been determined yet. TaqMan Genotyping Assay indicated that *CYP2B6* 449G was heterozygous with 499C in 12 individuals (2.6%), who were all Japanese (table 1). Direct sequencing of all the exons confirmed the results of TaqMan Genotyping Assay and showed that 8 subjects had 516GT, 785AG, and 1375AA genotypes; 3 had 516TT, 785GG, and 1375AA genotypes; and 1 had 516GT, 785AG, and 1375AG genotypes without any other mutation. Subcloning analysis of the PCR products confirmed that 499G always coexisted in the same allele with 516T and 785G (figure 1). Therefore, it was concluded that the novel haplotype containing 499C→G had 2 other single-nucleotide polymorphisms (SNPs): 516G→T and 785A→G. We formally registered this novel allele with the Human Cytochrome P450 Allele Nomenclature Committee, and it was designated "*CYP2B6 *26*" (http://www.cypalleles.ki.se/). With use of this nomenclature, the *CYP2B6* haplotype of the twelve 499C→G carriers were identified as eight **1/*26* heterozygotes, three **6/*26* heterozygotes, and one **23/*26* heterozygote (table 1). The allelic frequency of **26* was 1.3% in our study participants.

CYP2B6 *haplotype determination.* In 456 HIV-1–infected individuals, we determined the genotypes of 9 SNP positions (64C→T, 415A→G, 499C→G, 516G→T, 777C→A, 785A→G, 983T→C, 1375A→G, and 1459C→T) in *CYP2B6* (table 1). No *CYP2B6* genetic polymorphism was detected in 211 patients, and their haplotype was determined to be **1/*1*. The haplotypes

Table 1. *CYP2B6* haplotype and allele frequencies in study participants.

CYP2B6 status	*CYP2B6* genotype at nucleotide position								No. (%) of subjects	
	415	499	516	777	785	983	1375	1459	All[a]	Japanese
Haplotype:										
**1/*1*	AA	CC	GG	CC	AA	TT	AA	CC	211 (46.3)	205 (46.4)
**1/*2*	AA	CC	GG	CC	AA	TT	AA	CC	30 (6.6)	30 (6.8)
**1/*4*	AA	CC	GG	CC	AG	TT	AA	CC	43 (9.4)	42 (9.5)
**1/*5*	AA	CC	GG	CC	AA	TT	AA	CT	4 (0.9)	3 (0.7)
**1/*6*	AA	CC	GT	CC	AG	TT	AA	CC	104 (22.8)	101 (22.9)
**1/*23*	AA	CC	GG	CC	AA	TT	AG	CC	2 (0.4)	2 (0.5)
**1/*26*	AA	CG	GT	CC	AG	TT	AA	CC	8 (1.8)	8 (1.8)
**2/*4*	AA	CC	GG	CC	AG	TT	AA	CC	6 (1.3)	5 (1.1)
**2/*5*	AA	CC	GG	CC	AA	TT	AA	CT	1 (0.2)	1 (0.2)
**2/*6*	AA	CC	GT	CC	AG	TT	AA	CC	5 (1.1)	5 (1.1)
**4/*4*	AA	CC	GG	CC	GG	TT	AA	CC	5 (1.1)	5 (1.1)
**4/*6*	AA	CC	GT	CC	GG	TT	AA	CC	12 (2.6)	12 (2.7)
**5/*5*	AA	CC	GG	CC	AA	TT	AA	TT	1 (0.2)	1 (0.2)
**5/*6*	AA	CC	GT	CC	AG	TT	AA	CT	1 (0.2)	1 (0.2)
**6/*6*	AA	CC	TT	CC	GG	TT	AA	CC	19 (4.2)	17 (3.8)
**6/*26*	AA	CG	TT	CC	GG	TT	AA	CC	3 (0.7)	3 (0.7)
**23/*26*	AA	CG	GT	CC	AG	TT	AG	CC	1 (0.2)	1 (0.2)
Total									456	442
Allele:										
**1*	A	C	G	C	A	T	A	C	613 (67.2)	596 (67.4)
**2*	A	C	G	C	A	T	A	C	42 (4.6)	41 (4.6)
**4*	A	C	G	C	G	T	A	C	71 (7.8)	69 (7.8)
**5*	A	C	G	C	A	T	A	T	8 (0.9)	7 (0.8)
**6*	A	C	T	C	G	T	A	C	163 (17.9)	156 (17.6)
**23*	A	C	G	C	A	T	G	C	3 (0.3)	3 (0.3)
**26*	A	G	T	C	G	T	A	C	12 (1.3)	12 (1.4)
Total									912	884

[a] Including 442 Japanese, 8 other Asians (5 Thai, 2 Koreans, and 1 Filipino), 4 Hispanics, and 2 non-Hispanic whites.

of single-SNP carriers with 64CT, 785AG, 1375AG, and 1459CT were determined to be **1/*2*, **1/*4*, **1/*23*, and **1/*5*, respectively. Those of homozygous polymorphism carriers with 785GG only, 1459TT only, and both 516TT and 785GG were determined to be **4/*4*, **5/*5*, and **6/*6*, respectively. When the fact that **2* is the only allele harboring 64C→T is considered, patients with 64CT and 785AG; 64CT and 1459CT; and 64CT, 516GT, and 785AG were identified as **2/*4*, **2/*5*, and **2/*6* heterozygotes, respectively. Patients with both 516GT and 785GG genotypes but without other polymorphisms were determined to have **4/*6* heterozygotes. There were 104 patients (22.8%), including 101 Japanese, who held both 516GT and 785AG genotypes without other polymorphisms. There were 2 possible haplotypes, **1/*6* and **4/*9*, in this genotypic pattern. When the fact that **9* had not been reported in Japanese subjects was considered [11], we found that all 101 Japanese were **1/*6* heterozygotes. Haplotype analysis by subcloning of PCR products described above was performed in the 3 others, and their haplotype was determined as **1/*6*. One Japanese patient had 516GT, 785AG, and 1459CT genotypes without other polymorphisms, and there were 2 possible haplotypes, **1/*7* and **5/*6*, in this genotypic pattern. Because **7* had not been reported in Japanese subjects [11], the haplotype in this patient was determined to be **5/*6*. Overall, the allelic frequency of **6* was 17.9% in our study participants. The 415A→G, 777C→A, and 983T→C polymorphisms, which are the determinants of **8*, **3*, and **18*, respectively, were not observed in our subjects.

CYP2B6 *and EFV concentration.* We determined the *CYP2B6* haplotype in 251 patients at IMCJ and in 205 patients at the other 10 hospitals. Of the 251 genotype-analyzed patients at IMCJ, 101 were being treated or were beginning treatment with a standard dose of EFV during this study period (figure 2). Plasma EFV concentrations were measured in all 101 patients, including sixty-seven 516GG holders, twenty-eight 516GT holders, and six 516TT holders. To clarify the effect of the 516TT genotype, EFV concentration was also measured in ten 516TT holders undergoing treatment with the standard dose of EFV at other hospitals. The mean concentration (± SD)

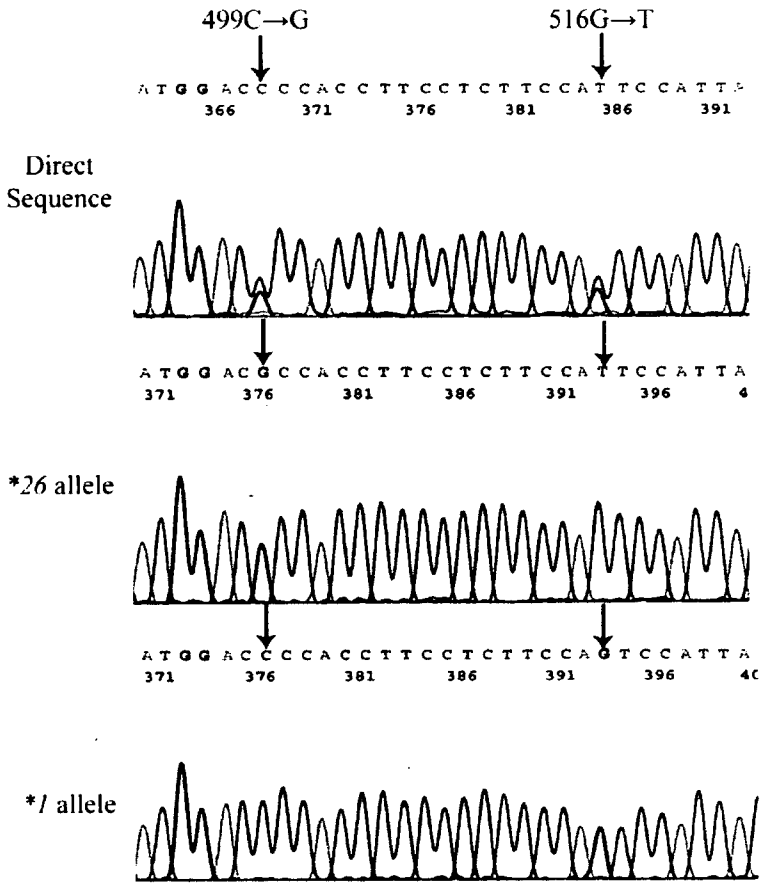

Figure 1. Direct *(top panel)* and subclonal *(middle and bottom panels)* sequences of *CYP2B6* in 499C→G carriers. The genotypes 499G, 516T, and 785G (not shown) exist in the same allele, newly designated as *"CYP2B6 *26."* The same results were obtained in all 9 patients with the 499CG, 516GT, and 785AG genotypes, and the patients were identified as eight **1/*26* carriers and one **23/*26* carrier. Although shown are the sense-strand sequences only, both strands were sequenced. Arrows indicate the variant nucleotide positions 499 and 516.

of EFV in all patients was 3740 ± 2800 ng/mL. When divided by the genotype of position 516, striking discreteness was observed (figure 3). All (95% CI 91.1%–100%) of the 16 carriers of 516TT genotype, including fourteen **6/*6* carriers and two **6/*26* carriers, had extremely high EFV concentrations (>6000 ng/mL). Their mean concentrations (9500 ± 2580 ng/mL) were many orders of magnitude higher than those of the other genotype carriers ($P < 10^{-4}$). There was no significant difference in EFV concentration between **6/*6* carriers and **6/*26* carriers. On the other hand, there were only 2 patients who had such high EFV concentrations among the other genotype carriers. One was a **1/*6* carrier (7140 ng/mL), and the other was a **1/*26* carrier (9710 ng/mL). Direct sequencing of all *CYP2B6* exons showed no polymorphism other than 499C→G, 516G→T, and 785A→G in these individuals. The mean concentrations of EFV of the twenty-eight 516GT carriers, including twenty-five **6*-heterozygotes (3320 ± 1240 ng/mL; $P < 10^{-4}$) and three **26*-heterozygotes (5470 ± 3840 ng/mL; $P < 10^{-4}$), were significantly higher than those of the sixty-seven 516GG genotype carriers (2450 ± 770 ng/mL). None (95% CI 0%–0.1%) of the 516GG carriers had a high EFV concentration (>6000 ng/mL). Considered together, it was concluded that high plasma EFV concentrations were associated with *CYP2B6 *6* and **26* and that *CYP2B6 *6/*6* and **6/*26* carriers had extremely high plasma EFV concentrations at standard dosage, without exception.

EFV dose reduction from 600 mg. To determine whether the EFV dose can be reduced in patients who have a high concentration while receiving the standard dose, a dose-reduction protocol was applied in 12 patients with high plasma concentrations (>6000 ng/mL [range, 6170–14,690 ng/mL]), including one **1/*26* heterozygote, nine **6/*6* homozygotes, and two **6/*26* heterozygotes. Before the dose reduction, plasma HIV-1 load was undetectable (<50 copies/mL) in all patients for >1 month with treatment of a standard antiretroviral regimen containing 600 mg of EFV. In these 12 patients,

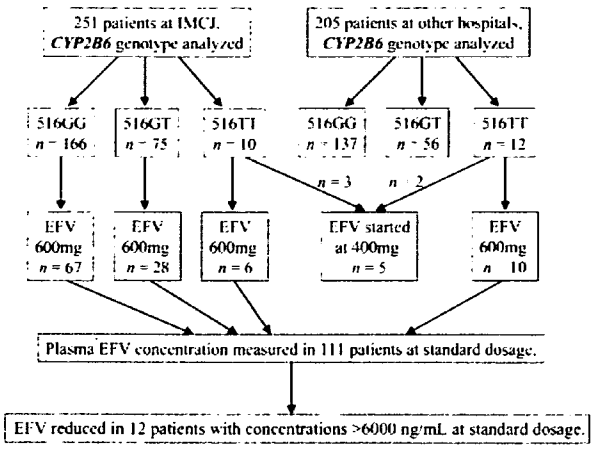

Figure 2. Flow diagram of study participants. The *CYP2B6* genotype was analyzed in 251 patients at the International Medical Center of Japan (IMCJ) and in 205 patients at other hospitals. Standard dosage of EFV was administered in 101 patients at IMCJ, including sixty-seven *CYP2B6* 516GG, twenty-eight 516GT, and six 516TT holders, whose EFV concentrations were measured. Ten 516TT holders at the other hospitals were administered standard dosages of EFV, and their EFV concentrations were also measured. A reduced-dose (400 mg) regimen of EFV was initiated in 5 other 516TT holders.

the EFV dose was reduced from 600 to 400 mg in 11 subjects and was further reduced to 200 mg in 7 of them who consented to further reduction. The plasma EFV concentrations decreased by approximately one-third (36%–46%), to 3720–6160 ng/mL, with dose reduction from 600 to 400 mg in 10 of 11 subjects, and further decreased by approximately one-half (51%–59%), to 1620–2960 ng/mL, with reduction from 400 to 200 mg in 6 of 7 subjects (figure 4). In one patient who had a markedly high EFV concentration (14,690 ng/mL) at the standard 600-mg dose, however, the concentration decreased unexpectedly by 69%, to 4500 ng/mL, with the reduction to 400 mg and further decreased by 82%, to 790 ng/mL, lower than the recommended range (>1000 ng/mL) [1], with the reduction from 400 to 200 mg. Therefore, the dose was increased in this patient back to 400 mg. In another patient who had reported severe dizziness during treatment with the standard dose (600 mg), the dose was reduced immediately to 200 mg at the patient's request. The plasma EFV concentration was also markedly high in this patient (14,360 ng/mL) during treatment with the standard dosage. However, it decreased by 83%, to 2410 ng/mL, with the dose reduction to 200 mg. Consequently, the final EFV dose was 400 mg in 5 subjects and 200 mg in 7 subjects. The determined dosage for each patient was continued for >6 months (the longest was 26 months for a patient who received the 200-mg dose), and the plasma HIV-1 load was continuously undetectable in all patients.

EFV initiation at 400-mg dose. Our analysis showed that *CYP2B6* **6/*6* and **6/*26* carriers had extremely high EFV concentrations, without exception (figure 3), and that dose reduction was possible in patients with high EFV concentration with retention of therapeutically effective anti–HIV-1 activity (figure 4). In the next phase of our study, we used an antiretroviral regimen containing a reduced dose (400 mg) of EFV in 5 EFV-naive patients (four **6/*6* homozygotes and one **6/*26* heterozygote). Before the introduction of low-dose EFV-containing regimen, the plasma HIV-1 loads had been undetectable during receipt of the previous protease inhibitor–containing regimen in all 5 patients. Their EFV concentrations were 4080–9450 ng/mL, and all such concentrations (95% CI, 99.5%–100%) were therapeutically adequate (>1000 ng/mL) at the 400-mg dose (figure 5). One **6/*6* homozygote developed severe dizziness, necessitating discontinuation of EFV-treatment at day 16. His EFV concentration was 5430 ng/mL. In one **6/*26* heterozygote, severe thrombocytopenia emerged, probably because of overdosage of rifabutin prescribed for the treatment of coinfection with *Mycobacterium intracellulare*, and EFV treatment was stopped at day 15. The EFV concentration was 5770 ng/mL. Two of the remaining 3 patients still had extremely high EFV concentrations (6760 and 9450 ng/mL) at the 400-mg dose, and their dose was subsequently reduced to 200 mg. The plasma EFV concentrations decreased to 2690 and 3660 ng/mL (i.e., by 60% and 61%, respectively). Consequently, 2 subjects

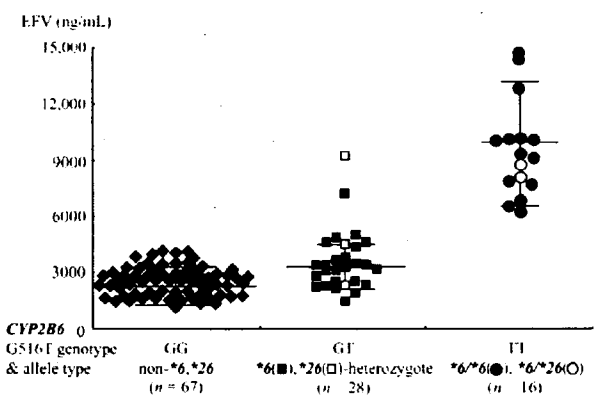

Figure 3. Plasma efavirenz (EFV) concentrations measured during EFV treatment with standard dose (600 mg). A total of 111 HIV-1–infected patients treated with EFV-containing regimens were divided into 3 groups on the basis of nucleotide genotype at *CYP2B6* position 516 (GG, GT, or TT), and their plasma EFV concentrations were compared. Blackened squares, **6* heterozygote with allele other than **26*; unblackened squares, *CYP2B6* 499C→G carriers (**26* heterozygote with allele other than **6*); blackened circles, **6* homozygote (**6/*6*); unblackened circles, *CYP2B6* 499C→G carriers (**6/*26* heterozygotes); blackened diamonds, other genotype carriers. Horizontal lines represent the mean (± SD) plasma EFV concentrations for each group.

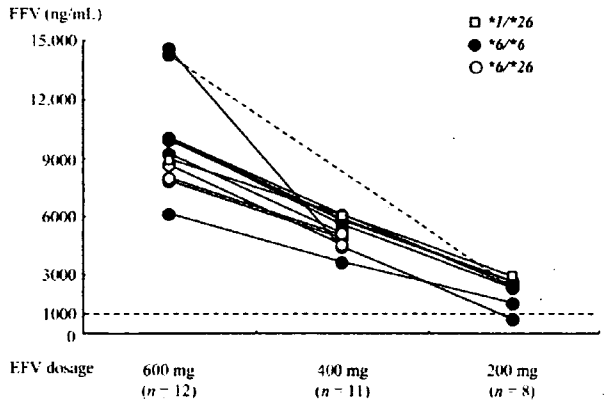

Figure 4. Dose reduction of efavirenz (EFV) in 12 patients whose concentrations were extremely high while receiving treatment with standard dose (600 mg). EFV dose was reduced from 600 to 400 mg in 11 patients and was further reduced, to 200 mg, in 7 patients. In one patient who had severe CNS symptoms while receiving treatment with standard dose, EFV dose was directly reduced to 200 mg (concentrations connected with a dotted line). The suggested minimum target concentration (1000 ng/mL) is indicated by the thin line.

discontinued the EFV-containing regimen, and 3 subjects continued low-dose EFV-containing regimen (400 mg for 1 patient and 200 mg for 2 patients). The low-dose regimen was continued for >6 months, and the plasma HIV-1 load was persistently undetectable in all 3 patients.

Improvement of CNS symptoms. As described above, the EFV dose was reduced from 600 to 400 and 200 mg as the final dose in 5 and 7 subjects, respectively (figure 4), and it was decreased from 400 mg as the initial dose to 200 mg for 2 other subjects (figure 5). To delineate the changes in CNS symptoms associated with the decrease in EFV concentration, a questionnaire survey of these 14 patients was conducted regarding 6 items: dizziness, strange dreams, depression, irritability, concentration problems, and sleep difficulty. More than 1 month after the dose had been reduced to the lowest dose, the patients were asked to judge the 6 CNS symptoms above at initial and final doses, with use of a 5-grade system ("none," "slight," "sometimes," "often," and "always"). Ten (71%) of the 14 patients had some of the aforementioned CNS symptoms during treatment with the initial dose (table 2). The most common symptom was dizziness (57%), followed by strange dreams (50%). Interestingly, all the symptoms improved after dose reduction in the 10 patients. Furthermore, dizziness and concentration problems disappeared during treatment with the final dose in one-half of the patients, although strange dreams and sleep difficulty were still reported by all the patients who had those difficulties at the initial dose. Finally, when the patients were asked whether they wanted to reincrease EFV to the previous dose, all 10 patients with CNS symptoms at the initial dose answered "no" (9 answered "absolutely no").

DISCUSSION

In this study, we identified a novel *CYP2B6* allele, **26*, which includes 499C→G, 516G→T, and 785A→G in 12 Japanese patients, and we showed that, without exception, all **6/*6* and **6/*26* carriers, all holding 516TT, had extremely high plasma EFV concentrations while receiving the standard dose (600 mg) [4]. In other reports, however, there were some exceptional subjects with 516TT who had normal concentrations of EFV, and the discreteness of the EFV concentration with the position 516 genotype was not as clear as it was in our patients [5–8]. This difference may be because some of the 516TT carriers had other *CYP2B6* alleles, such as **7* (containing 516G→T, 785A→G, and 1459C→T), **9* (containing 516G→T only), and **13* (containing 415A→G, 516G→T, and 785A→G). Those alleles could not be found in our subjects, and their effects on EFV concentration were not well described. Because numerous additional *CYP2B6* variants with impact on expression and/or function were recently reported [12–18], correct determination of *CYP2B6* haplotype seems indispensable for prediction of EFV plasma levels.

We reduced the EFV dose in 12 patients whose plasma EFV concentrations were extremely high while receiving the standard dose, and we initiated EFV treatment at a 400-mg dose in 5 EFV-naive **6/*6* and **6/*26* carriers. In most patients, the plasma EFV concentration decreased proportionally with the dose-reduction ratio. In 2 subjects, however, the concentrations decreased much more than expected, given the dose reduction

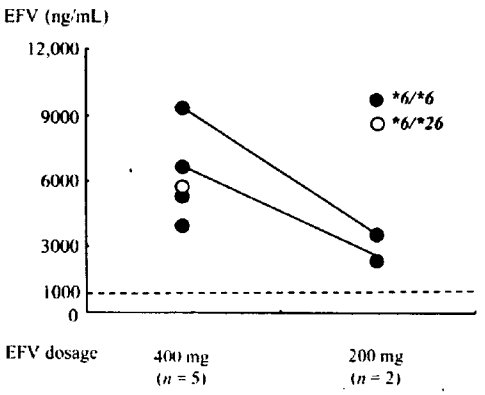

Figure 5. Introduction of low-dose efavirenz (EFV)-containing antiretroviral regimen to *CYP2B6 *6/*6* and **6/*26* carriers. Treatment was started in 4 EFV-naive carriers *CYP2B6 *6/*6* and one **6/*26* carrier, with 400-mg EFV-containing regimens. EFV dose was further reduced, to 200 mg, in 2 patients whose EFV concentrations were >6000 ng/mL while receiving treatment with the 400-mg dose.

Table 2. Changes in CNS-related symptoms after reduction of efavirenz dosage.

Symptom	No. (%) of subjects who reported symptom status during efavirenz treatment		
	Present[a] ($n=14$)	Improved[b]	Disappeared[b]
Dizziness	8 (57)	8 (100)	4 (50)
Strange dreams	7 (50)[c]	7 (100)[c]	0 (0)
Depression	5 (36)	5 (100)	1 (20)
Irritability	5 (36)	5 (100)	1 (20)
Concentration problem	4 (29)	4 (100)	2 (50)
Sleep difficulty	3 (21)	3 (100)	0 (0)
Any of the above	10 (71)[c]	10 (100)[c]	4 (40)

[a] Including the 4 grades "slight," "sometimes," "often," and "always" at the initial dosage. Includes 2 patients whose efavirenz treatment was originally 400 mg and was reduced to 200 mg.

[b] Percentage of those who initially reported "present."

[c] Including 1 patient whose efavirenz dose was originally 400 mg and was reduced to 200 mg.

ratio. Both of these patients had markedly high concentrations at standard dosage. Hasse et al. [19] reported a patient with excessively high plasma EFV concentration at standard dose, which decreased to one-thirtieth following dose reduction from 600 to 200 mg. Long-term exposure to such excessively high concentrations may induce CYP2B6 enzymatic expression in the liver, which could result in an unexpectedly large decrease in plasma EFV concentration by dose reduction if deinduction of the enzyme takes several weeks. At the 400-mg dose, the plasma concentrations of EFV were therapeutically adequate in all the treated **6/*6* and **6/*26* carriers in this study. Regarding the reduced dose, it is noteworthy that a phase II study during EFV development supported the use of a lower dose [20]. The same study indicated that the 600-mg dose of EFV is associated with a high rate of adverse events that could lead to discontinuation, which suggests that the lower dose of 400 mg may be almost as effective without the high discontinuation rate. In the present study, associated with the dose-reduction regimen, a significant number of patients experienced improvement of CNS symptoms, which was unexpected on the basis of previous reports [5, 21, 22]. Interestingly, some of these patients did not appreciate their clinical state and considered themselves to have no CNS-related symptoms during the standard-dose treatment. However, after the dose reduction, they reassessed the status and evaluated symptoms during the treatment with the standard dose as associated with CNS symptoms and indicated that the reduced dose of EFV relieved them of such symptoms. Because EFV-treated patients often stick to the regimen, previous reports of symptom questionnaires conducted during the standard treatment might have underestimated the EFV-associated CNS symptoms [5, 21, 22]. However, this finding might be confounded by placebo effect, because the patients were told that their EFV levels were high while receiving the initial dose and decreased throughout the dose-reduction protocol. Because of this possible placebo effect, a double-blind, placebo-controlled study would best address this question.

EFV dose reduction and initiation of EFV treatment at reduced dose is possible with therapeutic anti–HIV-1 potency retained in *CYP2B6 *6/*6* homozygotes and **6/*26* heterozygotes, which could relieve the patients of the EFV-associated CNS symptoms. It may also decrease the risk of development of EFV-resistant HIV-1 after mandatory treatment discontinuation, such as abdominal surgery [23], and reduce the treatment cost, an important issue in developing countries [24]. After dose reduction, however, careful monitoring is necessary until larger studies confirm the safety of reduced dose in such specific genotype carriers.

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Potential conflicts of interest. All authors: no conflicts.

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Original article

Integration of HIV-1 caused STAT3-associated B cell lymphoma in an AIDS patient

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Abstract

Signal transducer and activator of transcription 3 (STAT3) is a DNA-binding transcription factor activated by multiple cytokines and interferons. High expression of STAT3 has also been implicated in cancer and lymphoma. Here, we show a case of B cell lymphoma in which a defective human immunodeficiency virus 1 (HIV-1) integrated upstream of the first STAT3 coding exon. The lymphoma cells with anaplastic large cell morphology formed multiple nodular lesions in the lung of an acquired immunodeficiency syndrome (AIDS) patient with Kaposi's sarcoma. The provirus had a 5′ long terminal repeat (LTR) deletion, but the 3′ LTR had stronger promoter activity than the STAT3 promoter in reporter assays. Immunohistochemistry showed increased expression of STAT3 in the nuclei of lymphoma cells. Transfection of STAT3 resulted in transient cell proliferation in primary B cells in vitro. Although this is a very rare case of HIV-1-integrated lymphoma, these data suggest that up-regulation of STAT3 caused by HIV-1 integration resulted in the development of B cell lymphoma in this special case.

Keywords: HIV-1; Integration; AIDS-related lymphoma; STAT3

1. Introduction

Malignant lymphoma is an important complication of patients with acquired immunodeficiency syndrome (AIDS). A large part of AIDS-related lymphomas are of B cell linage, and positive for Epstein–Barr virus (EBV) or Kaposi's sarcoma-associated herpesvirus (KSHV) [1–4]. Since human immunodeficiency virus 1 (HIV-1) is not usually detected in AIDS-related lymphoma cells, HIV-1 infection plays an indirect role in lymphomagenesis by impairing host immune surveillance. However, proviral DNA can either disrupt expression of tumor suppressor genes or enhance expression of cellular oncogenes. Alternatively, retroviral promoters can integrate into the host genome in such a manner that expression of a nearby oncogene is enhanced by a strong promoter within the proviral 3′-long terminal repeat (3′LTR). In humans, abnormal T cell proliferation following gene therapy for severe combined immunodeficiency resulted from retroviral integration into the intron of the *LMO2* proto-oncogene [5].

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In AIDS patients, some cases of lymphomas had HIV-1 integration within the *fur* gene, just upstream from the *c-fes/fps* proto-oncogene [6]. That report, however, did not investigate the functional effect of this integration event. These observations suggest that HIV-1 may contribute directly to lymphomagenesis by inserting an active promoter into a cellular oncogene [6]. In the present study, we report a case of AIDS-related lymphoma in which HIV-1 integrated upstream of the STAT3 gene. The association of HIV-integration and lymphomagenesis was investigated.

2. Materials and methods

2.1. Samples

Lymphoma tissues in the lung of a patient with HIV-1 infection were obtained at autopsy. Formalin-fixed pathological samples of lymphoma, including nine unrelated cases of AIDS-related lymphoma and 15 cases of non-Hodgkin lymphoma in HIV-1-uninfected individuals, were studied. All samples were obtained with informed consent according to the Declaration of Helsinki. The study protocol was approved by the institutional review board of National Institute of Infectious Diseases (Approval No. 93).

2.2. Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as described before [7,8]. Primary antibodies were: anti-CD3 (Dako, Copenhagen, Denmark), CD20 (Dako), CD30 (Dako), CD45 (Dako), CD45RO (Dako), CD79a (Dako), CD138 (Serotec, Oxford, UK), and $p80^{NPM/ALK}$ (Nichirei, Tokyo, Japan), STAT3 (sc8019, Santa Cruz Biotechnology, Santa Cruz, CA), pSTAT3 (sc8059, Santa Cruz), KSHV-encoded LANA [8], and vIL-6 [7] antibodies. In situ hybridization for EBERs was performed as described before [9].

2.3. PCR and DNA sequences

PCR detection for KSHV-encoded open reading frame (ORF) 26, EBV W region, HIV-1 V3, and β-globin gene was performed as described previously [9,10]. For PCR amplification of HIV-1 3′LTR and STAT3 junction, HIV3LTR-F (5′-TCTGAGCCTGGGAGCTCTCT-3′, 9561–9580 in GenBank K03455) and Stat3intron-R (5′-AGTGCATGGCACATAACAGA-3′, 41131–41150 in GenBank AY572796) were used. For amplification of HIV-1 5′LTR and STAT3 junction, 6 reverse primers of 5′LTR (55R 5′-TCAGGGAAGTAGCCTTGTGTGTGGT-3′, 78R 5′-GCCCTGGTGTGTAGTTCTGTCAATC-3′, 348R 5′-GAAAGTCCCCAGTGGAAAGTCCCTT-3′, 495R 5′-GCAGTGGGTTCCCTAGTTAGCC-3′, 563R 5′-TTACCAGAGTCACACAACAGACGGG-3′, and 612R 5′-CACTGCTAGAGATTTTCCACACTGAC-3′), and a reverse primer positioning between 5′LTR and gag (676R 5′-CGAGTCCTGCGTCGAGAGATCTCCT-3′) were used with a forward primer of Stat3-intronF2 (5′-CATTTTTCTTTCCTTCTCTGTTGTC-3′, 40881–40905 in GenBank AY572796). These primers for HIV-1 were designed based on the sequence of HIV-1 IIIB (GenBank K03455).

2.4. Cloning of HIV-1 integration sites

The methods used were essentially as described for the Gene Walker Kit (BD Clontech, Palo Alto, CA). Lung tumor DNA was cleaved with four different blunt cutting enzymes (*Dra*I, *Eco*RV, *Pvu*II and *Ssp*I). Gene specific primers for HIV-1 LTR were 5′-ACCACACACAAGGCTACTTCCCTGA-3′ (GSP-1) and 5′-AAGGGACTTTCCACTGGGGACTTTC-3′ (GSP-2).

2.5. Real-time PCR

Copy numbers of HIV-1 integration site and STAT3 gene were measured with real time PCR as described previously [11]. Two probe and primer sets were used (Set 1: forward primer: 5′-CTAGAGATCCCTCAGACCATTTTAGTC-3′, reverse: 5′-AAAAGTATAAATGAGGATCCAGGAAGAT-3′, probe: 5′-6FAM-TGTGGAAAATCTCTAGCAGAATCTCAGG-TAMRA-3′; Set 2: forward primer: 5′-GCAGCTTGACACACGGTACCT-3′, reverse: 5′-AAACTGCCGCAGCTCCATT-3′, probe: 5′-6FAM-AGCAGCTCCATCAGCTCTACAGTGACAGC-TAMRA-3′).

2.6. Plasmids

For the promoter assay, genes of the HIV-1 3′LTR, STAT3-intron (40951–41959 of GenBank AY572796), and STAT3-promoter (1–1998 of GenBank AY572796) were amplified from DNA of the HIV-1-integrated lymphoma using the LTR-*Mlu*I-F, 5′-GAG*ACGCGT*TGGAAGGGCTAATTCACTCCC-3′ and LTR-*Xho*I-R, 5′-GTG*CTCGAG*TGCTAGAGATTTTCCACACT-3′, the Intron-*Mlu*I-F, 5′-GAG*ACGCGT*GAATCTCAGGCAGATCTTCC-3′ and Intron-*Xho*I-R, 5′-CAC*CTCGAG*CCTGCTAAAATCAGGGGTCCC-3′, and, the Stat3prom-*Mlu*F1, 5′-GAG*ACGCGT*ACCCATAGTCGCAGAGGTAGA-3′ and Stat3prom-*Xho*R1, 5′-GAG*CTCGAG*CGCTGAATTACAGCCCCTTCA-3′, respectively. Enzyme sites are indicated in italics. A fragment of the HIV-1 3′LTR was amplified also from HIV-1 pNL4-3 (GenBank AF324493). The PCR product was subcloned into *Mlu*I-*Xho*I site of pGL3-basic vector (Promega, Madison, WI). For the STAT3-expression plasmid, STAT3 cDNA was amplified from the mammalian gene collection-human (MGC-1607, American type culture collection, Manassas, VA) using forward primer (STAT3-HpaI-F10 5′-CACCGTTAACGGATCCTGGACAGGCACCC-3′) and reverse primer (STAT3-R24 5′-CATGTCAAAGGTGAGGGACTCAAA-3′). The PCR product was TA cloned using pcDNA 3.1 Directional TOPO Expression kit (Invitrogen, Carlsbad, CA). For cell proliferation experiment, the STAT3 expression vector was digested with *Hin*dIII and *Eco*RV and ligated into *Bsm*BI and *Eco*RV sites of pMACS 4-IRES.II vector, which is a bicistronic expression vector containing multiple cloning site followed by an internal ribosome entry site (IRES) element

from encephalomyocarditis virus and the truncated (non-functional) CD4 cDNA (Miltenyl Biotec, Auburn CA).

2.7. Promoter assay

Plasmids were transiently transfected into HeLa cells with a renilla reporter gene construct using Lipofectamine Plus (Invitrogen). Luciferase activity was measured with a dual luciferase assay system (Promega). In the HIV-1-Tat (+) group, an HIV-1-Tat expression vector, kindly provided by Dr. Kenzo Tokunaga, National Institute of Infectious Diseases, Tokyo, Japan, was cotransfected.

2.8. DNA methylation analysis

Methylation of the cytosine residue of the CpG site was analyzed by the bisulfite genomic sequencing method, as described previously [12]. The primer pair for selective analysis was as follows: sense primer, 5′-TATAAACCAGCATGG GATGGATGA-3′; antisense primer, 5′-CCCAGGCTCGGA TCTGGTCTAACC-3′.

2.9. Cell proliferation assay for primary lymphocytes

Primary B cells were negatively selected from whole blood of healthy volunteers using RosetteSep B cell enrichment (StemCell Technology, Vancouver, BC, Canada) [13]. Cell proliferation assay was performed using BrdU Cell proliferation ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN).

3. Results

3.1. HIV-1 was concentrated in lymphoma cells in a case of AIDS-related lymphoma

A 59-year-old, homosexual, HIV-1-positive male with a CD4 cell count of 6/mm^3 showed high fever and multiple KS skin lesions. Computed tomography scanning revealed multiple nodules in the lung (Fig. 1A). Despite treatments with antibiotics and combined chemotherapy, with intensive care, he died 30 days after admission. The clinical course of the patient was also reported previously [14]. At autopsy, multiple nodules were present in the lung (Fig. 1B). Histologically, these nodules were composed of large atypical cells with anaplastic large cell morphology infiltrating into interstitial and alveolar areas in the lung tissue (Fig. 1C). Immunohistochemistry demonstrated that the tumor cells were CD3$^-$, CD20$^-$, CD30$^+$, CD45$^+$, CD45RO$^+$, CD79a$^-$, CD138$^-$, and p80$^{NPM/ALK-}$, suggesting that the lung tumor was composed of lymphoma cells (Fig. 1D and data not shown) [14]. Southern blot hybridization of DNA extracted from the lung tumor with an immunoglobulin junction hinge (JH) probe demonstrated immunoglobulin gene rearrangement, confirming a B

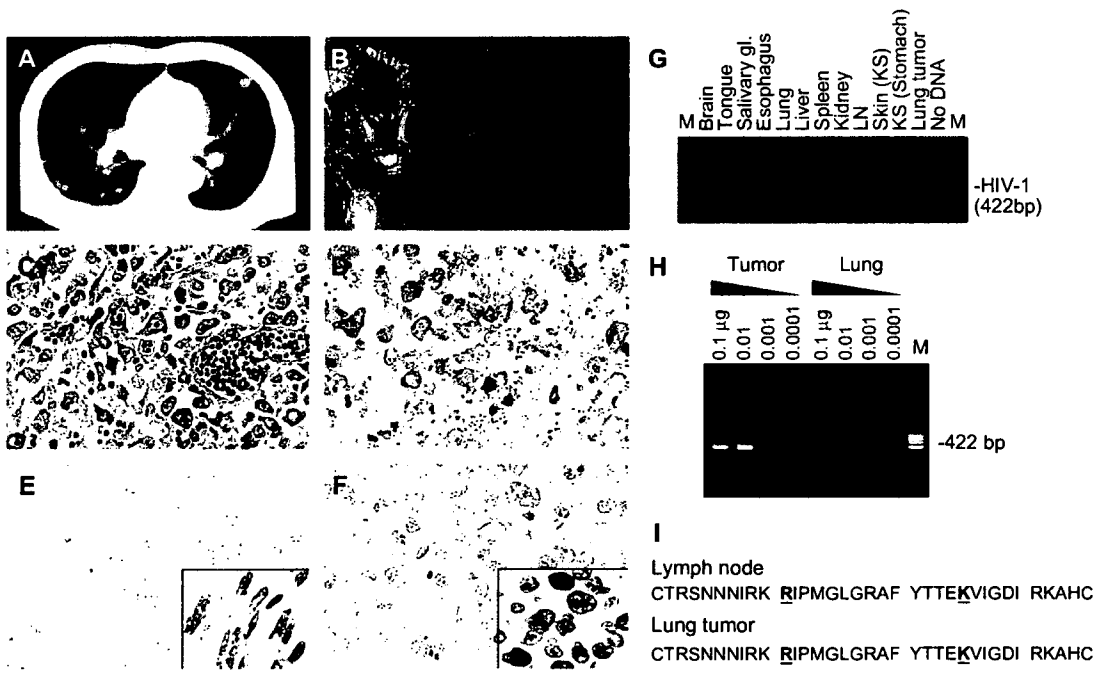

Fig. 1. Pathological findings of tumors in the lung of a patient with AIDS. CT scan (A), macroscopic view (B) and Hematoxylin and eosin staining (C) of the lung tumor. (D) Immunohistochemistry of CD45RO. (E) Immunohistochemistry for KSHV-LANA in the lung tumor cells. Inset shows gastric KS cells from the patient. (F) In situ hybridization for EBV-EBER in the lung tumor cells. Inset shows a positive control of EBV-positive lymphoma from an unrelated patient. (G) PCR detection for HIV-1 V3 region in various organs of the patient. LN, lymph node; M, DNA molecular weight marker (pBR322/*Hae*III). (H) Semi-quantitative PCR for HIV-1. DNA quantities are indicated at the top of the panel. DNA extracted from the lung tumor and surrounding lung tissues was tested. (I) Predicted amino acid sequence of HIV-1 gp120 V3 loop of HIV-1 amplified from the lymph node and lung tumor by PCR. Positions 11 and 25 are indicated by bold letters with underlines. DNA sequences are deposited in GenBank under accession numbers DQ116951 to DQ116954 (HIV-1 envelope from LN and lung tumor).

cell linage (data not shown). Since KS lesions were found in the oral cavity, stomach, sole and some lymph nodes at autopsy, we examined KSHV positivity in the lymphoma (lung tumor). KSHV-encoded ORF26 was amplified in both gastric KS lesions and lung tumor by PCR (data not shown). However, immunohistochemistry demonstrated that expression of KSHV LANA was very weak or absent in the lymphoma cells, whereas KS cells in the stomach strongly expressed LANA (Fig. 1E). Immunohistochemistry also demonstrated that the lung tumor cells were negative for KSHV-encoded vIL-6 (data not shown). The lymphoma cells were positive for EBV by PCR (data not shown), but in situ hybridization failed to detect EBERs (Fig. 1F). Thus, these data suggest that KSHV and EBV were present in the lymphoma at low copy numbers. Surprisingly, HIV-1 DNA was detected in the lymphoma cells by PCR, but not in other organs besides the lymph nodes (Fig. 1G). Semi-quantitative PCR revealed that there was a 100-fold higher copy number of HIV-1 DNA from the lymphoma than from surrounding lung tissue (Fig. 1H). PCR products of HIV-1 V3 region were TA-cloned and each 10 clones were sequenced. Although two (clones L2 and T3) and three (clones T1, T3, and T6) kinds of sequences were obtained from the lymph nodes and lymphoma, respectively, all sequences coded the same amino acid sequence in the V3 loop (net charge = + 7). Basic amino acids at positions 11 and 25 of the gp120 V3 loop and a high positive net charge strongly suggest that fusogenic X4 viruses were detected in the lymphoma cells and lymph nodes (Fig. 1I) [15].

3.2. HIV-1 integration in the STAT3 gene

A high copy number of HIV-1 in the lymphoma suggested integration of HIV-1 into the genome of lymphoma cells. Genome walking PCR produced a 400 bp fragment which contained a 300 bp fragment with >99% identity with the HIV-1 IIIB 3′LTR sequence (GenBank K03455) and A 40 bp genomic segment just before the first coding exon of STAT3 (Fig. 2A). PCR using primers in HIV-1 3′LTR and STAT3-intron yielded an independent amplicon with HIV-1 3′LTR and the predicted STAT3 genomic sequences from DNA of the lymphoma cells (Fig. 2B). These data confirmed that HIV-1 had integrated into the intervening sequence just before the first coding exon of STAT3. PCR using a primer pair binding to the STAT3 intron and upstream of HIV-1 gag demonstrates that the 5′LTR of the integrated HIV-1 was truncated (Fig. 2C). The sequence analysis revealed that the integrated HIV-1 lacked a fragment at the position of 1–587 in the 5′LTR (Fig. 2A,D, GenBank AF538307). Compared with the sequence of the 3′ integration site, HIV-1 integration resulted in duplication of the cellular 5 bp (GAATC) and addition of a dinucleotide at the integration site by HIV-1 integrase, which is commonly seen among retrovirus integrases [16,17]. Consequently, the integration event was produced by a defective virus (Fig. 2A,D). The absence of p24-staining of the tumor is consistent with this conclusion (data not shown).

3.3. Copy number of the integrated HIV-1 in the lymphoma tissue

Generally, pathological tissues obtained from lymphoma lesions contain not only lymphoma cells, but also surrounding CD4-positive T cells or alveolar macrophages. Although immunohistochemistry demonstrated no or rare CD4-positive cells in the lymphoma tissue, we tried to determine a copy number of the integrated HIV-1 in the lymphoma tissue by a real time PCR targeting genes near the integration site to deny the possibility that HIV-1 integration was originated in the contaminated CD4-positive cells (Fig. 3). A fragment of HIV-1-integration site was amplified at 12,570 copies/100 ng of DNA by the real time PCR, whereas exon 1 of STAT3 gene was amplified at 121,597 copies/100 ng. Since each cell has two copies of STAT3 gene on two alleles, these data suggest that HIV-1 integration occurred about 20% of the population that the DNA was extracted from. As shown in Fig. 1C, the lymphoma tissue contained many cells other than lymphoma cells, such as alveolar epithelial cells, macrophages, and endothelial cells. However, CD4-positive T cells were rare in the tissue, and the HIV-1 was X4 virus. Therefore, these data suggest that the HIV-1 might be detected from lymphoma cells, not from contaminated T cells or macrophages, and integrate into more than 20% of the lymphoma cells.

3.4. Promoter activity and methylation of HIV-1 3′LTR

LTRs of HIV-1 usually have a promoter activity in HIV-1-infected T cells and macrophages [18]. To investigate if the HIV-1 3′LTR contained a functional promoter, we constructed a plasmid containing the patient's HIV-1 3′LTR or upstream intron sequence of STAT3 before a luciferase reporter gene. Transfection of the plasmid to HeLa cells revealed that the sequence of 3′LTR derived from the patient had significant promoter activity at a similar level to that of 3′LTR in HIV-1 NL4-3, but the upstream intron sequence of STAT3 did not (Fig. 4A). 3′LTR was a stronger promoter than the STAT3 promoter derived from the patient. Cotransfection with a plasmid expressing HIV-1-Tat enhanced the activity of the patient's 3′LTR 31-fold, whereas the activity of the STAT3 promoter was not enhanced. These data suggest that the HIV-1 3′LTR contains promoter activity. It is known that DNA CpG methylation inactivates retroviral promoter including HIV-1 LTR [12,19]. However, a bisulfite genomic sequence revealed that the fragment of HIV-1 3′LTR did not have any CpG or non-CpG methylation in the DNA extracted from the lymphoma (Fig. 4B,C). These data suggest that methylation might not reduce or inhibit the transcriptional activity of HIV-1 3′LTR in the HIV-1-integrated lymphoma cells.

3.5. Expression of STAT3 in the HIV-1-integrated lymphoma

We investigated expression of STAT3 in the case of HIV-1-integrated lymphoma. Immunohistochemistry demonstrated a high level of STAT3 expression predominantly in the nuclei