and, therefore, our strategy of adopting FANTOM3 was feasible and allowed us to perform a genomic analysis by searching the highly conserved genomic region proximal to the transcription start sites. Such conserved regions were supposed to be critical *cis*-regulatory elements that are actually gene promoters. Here, we could demonstrate that this genome-based analysis lead to the identification of a highly conserved genomic region proximal to the pri-miR-223 transcription start sites that is the actual core promoter and most likely determines myeloid-specific expression of this miRNA. Further, we could identify PU.1 and C/EBP transcription factor-binding motifs, suggesting our approach might prove useful for studying the regulation of other miRNAs. Because gene-expression pattern is regulated by the unique combination of tissue-specific transcription factors, knowing that a specific set of transcription factors binds to a conserved genomic element would give clues for the tissue- and/or cellular-expression pattern of a specific miRNA. Our strategy would also be prominent in this view since many miRNAs have not yet been precisely characterized for their expression patterns and regulatory mechanisms.

Upon the homology search for cDNAs encoding pre-miRNAs, we identified a total of 42 complete pairings of cDNA-pre-miRNAs and practically complete pairings for 33 miRNAs. Further screening for miRNAs with highly conserved genomic regions around the transcription start sites resulted in the identification of ten miRNAs that fit the criterion. Since hundreds of miRNAs have been cloned to date (Bartel and Chen, 2004; Bentwich et al., 2005), this number appears to be very small. However, we should note that cDNAs as putative pri-miRNA candidates presented in this study are for so-called "exonic" miRNAs (Kim and Nam, 2006). There are many miRNAs derived from intronic regions of mRNA-like RNAs that are designated as "intronic" miRNAs (Kim and Nam, 2006). Indeed, a preliminary screening for pri-miRNAs of intronic miRNAs that used a similar approach identified candidate cDNA clones for more than 100 miRNAs (Y.F. and T.F. unpublished data), with many of them bearing conserved genomic elements.

In summary, by exploiting the combination of bioinformatics and biological approaches, we found a transcriptional mechanism for the myeloid expression of mouse miR-223 that is conserved in human miR-223 regulation. Our study thus proposes an evolutionarily conserved mechanism for miR-223 expression, and we propose an alternative to the previous model of Fazi et al. (2005). Further investigation will have to clarify which of the two mechanisms contributes to the regulation of miR-223, whereby the two need not be mutually exclusive.

## EXPERIMENTAL PROCEDURES

### Bioinformatics

#### *Extraction of mRNA-like RNAs Encoding miRNA Precursor Sequences*

A total of 337 precursor sequences of *Mus musculus* miRNA were obtained from the miRBase Sequence Database, Release 8.1 (http://microrna.sanger.ac.uk/sequences/ftp.shtml; Griffiths-Jones, 2004). The Riken FANTOM3 database provides mouse nonredundant, full-length cDNA sequences, and a total 102,802 of those sequences were downloaded (ftp://fantom.gsc.riken.jp/FANTOM3/). A homology search was exhaustively conducted between miRNA precursor sequences and cDNA sequences by using BLAST 2.2.12 with a cutoff value of $E = 1.0e^{-5}$. Among resultant cDNA-pre-miRNA pairs (294 pairs for 61 miRNAs), those with a perfect homology were extracted.

#### *Analyses of Transcription Factor-Binding Sites and Genome Conservation*

To identify the transcription start site, cDNA sequences containing pre-miRNA were mapped to the mouse genome, referring to the NCBI database (http://www.ncbi.nlm.nih.gov). Phylogenetic conservation from 250 bp upstream sequence of each 5' boundary was analyzed in a Mouse BLAT search of the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway). Putative binding sites for transcription factors of conserved genomic regions were explored by conducting the Transcription Element Search System (http://www.cbil.upenn.edu/tess/index.html).

### Cell Culture, Transfection, and DNA Constructs

Conditions of cell cultures and transfections are detailed in the Supplemental Data. Details of plasmid constructions are also described in the Supplemental Data.

### Chromatin Immunoprecipitation Assay

ChIP was performed with Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, Charlottesville, VA) according to the manufacturer's recommendation. Antibodies used for ChIP were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Genomic regions corresponding to promoters of mouse pri-miR-223, Cathepsin C, and c-Myc were amplified by PCR using specific primers shown in Table S1.

### Quantitative Real-Time RT-PCR

Total RNA was isolated from cells using mirVana miRNA Isolation Kit (Ambion) or miRNeasy mini kit (Qiagen) with the protocol specialized for total RNA purification. Relative quantification of human pri-miR-223 was performed by real-time PCR with SYBR green I. Level of mouse pri-miR-223 was determined with Taqman technology. Primers amplifying the mouse pre-miR-223 region and a specific Taqman probe were designed with PrimerExpress software (Applied Biosystems). Taqman MicroRNA Assay (Applied Biosystems) technology with a mature miR-223-specific probe and RT and PCR primers were used for quantification of mature miR-223, according to the manufacturer's instructions. Amplification of HPRT mRNA (for pri-miR-223; Applied Biosystems) and U6 small RNA (for mature miR-223; Applied Biosystems) was done with each experimental sample as an endogenous control to account for differences in the amount and quality of total RNA added to each reaction. All primers and the Taqman probe are shown in Table S1.

(D) Effects of synthetic siRNA-mediated PU.1 or C/EBPα knockdown on ATRA-induced pri-miR-223 promoter activity. NC indicates negative control. Data are shown with mean ± SD of triplicate measurements.

(E and F) Knockdown of PU.1 (left) or C/EBPα (p42 isoform; right) by corresponding shRNA vectors was confirmed by western blotting (E). Note that there is significant upregulation of PU.1 upon ATRA-treatment as reported previously (Mueller et al., 2006). A portion of each sample was subjected to measurement of the pri-miR-223 level (F). Transfectants were enriched as described in the Supplemental Data. Data in (F) were shown with mean ± SD of triplicate assays.

### RACE
Total RNA from human bone marrow was obtained from Clontech and used for 5′ RACE. For isolation of total RNA from NB4 cells and to avoid any loss of small RNA fractions, we used the mirVana miRNA Isolation Kit or the miRNeasy mini kit (Qiagen) with the protocol specialized for total RNA purification. The 5′ RACE was performed using the GeneRacer kit (Invitrogen). RACE products were obtained by PCR using TaKaRa Ex Taq (TAKARA Bio. Inc., Tokyo, Japan) with the sense primer provided in the GeneRacer kit and the antisense primer specific for human *miR-223* gene listed in Table S1 and were sequenced to determine 5′ ends of human pri-miR-223. For sequencing, RACE products were separated on agarose gels by electrophoresis, and individual bands were cloned using pGEM-T Easy Vector Systems (Promega).

### RNA Interference
Knockdown of hPU.1 and hC/EBPα by RNAi was accomplished by transfections of either duplex siRNAs (for the promoter-reporter assay) or shRNA expression vectors. Duplex siRNAs were synthesized by Japan Bio Services Co., LTD. (Saitama, Japan). Both synthetic and vector-based siRNAs target the same sites in corresponding transcription-factor mRNAs, and the sequences are shown in Table S1. The RNAi target sites for hPU.1 and hC/EBPα have been evaluated empirically and literally (Mueller et al., 2006). AllStars Negative Control siRNA was purchased from Qiagen and was used as the negative-control synthetic siRNA.

### Western Blotting
Western blotting was performed as described previously (Fukao et al. 2002) and is also detailed in the Supplemental Data. Affinity-purified rabbit anti-PU.1 and -C/EBPα antisera were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was purchased from Sigma.

### Supplemental Data
Supplemental Data include Experimental Procedures, References, four figures, and one table and can be found with this article online at http://www.cell.com/cgi/content/full/129/3/617/DC1/.

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# Vpr in Plasma of HIV Type 1-Positive Patients Is Correlated with the HIV Type 1 RNA Titers

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## ABSTRACT

**Vpr, an accessory gene product of HIV-1, has been reported in the plasma of HIV-1-positive patients, and exogenous Vpr induces the reactivation of viral production from latently infected cells and the apoptosis of T cells *in vitro*. These observations imply that Vpr is important in AIDS development, but the clinical relevance of the findings cannot be evaluated fully because the actual plasma Vpr concentration in HIV-1-positive patients is unknown. Here we generated two monoclonal antibodies against different portions of Vpr and successfully identified Vpr as a 14-kDa protein in HIV-1-positive patients. Semiquantitative analysis using a recombinant Vpr revealed that the concentration of Vpr in patient plasma was ~0.7 nM (10 ng/ml). Cross-sectional analysis of 52 HIV-1-positive patients revealed that the presence of Vpr detected in 20 patients was positively correlated with HIV-1 RNA copy number ($p < 0.03$), but not with the number of $CD4^+$ T cells. This is the first report demonstrating the actual amount of Vpr in HIV-1-positive patients, and the possible linkage of Vpr and viral titers indicates that it is important to continue to carry out the sequential analysis of Vpr, especially in clinical courses of HIV-1-positive patients. The threshold of viral titers, where Vpr appears in the patients' plasma, if present, contributes to better understanding the role of Vpr in AIDS pathogenesis.**

THE ADOPTION OF ANTIRETROVIRAL THERAPY (ART) has improved the prognosis of HIV-1-positive patients.[1] However, the complete elimination of the virus from patients receiving ART is estimated to take more than 60 years.[2] One factor that may be responsible for this problem is that HIV-1 infects macrophages, latent viral reservoirs[3] from which recurrent viral production is induced by various factors.[4] *Vpr*, an accessory gene of HIV-1, encodes a virion-associated 14-kDa protein that may be critical for the primary infection of macrophages.[5–7] Vpr also induces the reactivation of viral reproduction from latently infected cells. The presence of Vpr in the sera of HIV-1-positive patients, along with the induction of viral reproduction by exogenous Vpr,[8,9] implies that Vpr is actively involved in AIDS development. However, it is necessary to determine the concentration of Vpr in patient plasma samples to correctly evaluate the clinical significance of data obtained from *in vitro* experiments. In the current study, we successfully detected Vpr in patients' samples.

The protocol of this study was approved by the ethics committees of the International Medical Centre of Japan, Nara Medical University, Shizuoka Children's Hospital, and five other hospitals in collaboration with Shizuoka Children's Hospital. Blood plasma samples and peripheral blood were obtained from patients who had given informed consent after the experiment was explained to them. Clinical data on 14 outpatients at Nara Medical University, who were enrolled in the initial study, are

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summarized in Table 1. For the second study, samples from an additional 38 patients were analyzed. The median numbers of HIV-1 RNA copies (32,289.3 copies/ml), CD4$^+$ T cells (449.4 copies/ml), and total white blood cells (5049.0 cells/ml) were determined in all 52 patients. Control healthy plasma samples were obtained from Teragenix Corporation (Kokusai Bio, Tokyo). A recombinant Vpr protein (rVpr) was first prepared as a fusion protein with glutathione *S*-transferase (GST) expressed by pGEX6-P-1, and purified according to the manufacturer's protocol (GE Healthcare Bio-Sciences, Piscataway, NJ). The purified rVpr appeared as a single band on Coomassie brilliant blue staining (supplementary information 1a: SI-1a). Two mouse monoclonal antibodies, 8D1 (IgG2a) and C217 (IgG2b), were generated by immunization with a full-length Vpr peptide, chemically synthesized based on the prototype NL4-3[10] (Osaka Peptide Institute, Osaka), and a synthetic 18-mer amino acid peptide encompassing its carboxy (C)-terminal region (Wako Pure Chemical Industries, Tokyo, Japan), respectively. An enzyme-linked immunosorbent assay (ELISA) was based on 8D1, as the primary antibody, and a purified rabbit IgG antibody, raised against the peptide of the C-terminal 18 amino acids of Vpr (IBL, Fujioka, Japan), as the second antibody. Although the Vpr-ELISA could clearly detect purified rVpr (SI-1b), we found that the system occasionally detected one or more cross-reacting peptide in healthy persons (data not shown). Therefore, we decided to carry out a semiquantitative analysis using immunoprecipitation-Western blotting (IP-WB) analysis, with rVpr quantified by ELISA as the standard. For the IP-WB analysis, 0.5 mg of C217 was bound to Protein G Sepharose (GE Healthcare Bio-Sciences). Each 200 $\mu$l of plasma was first treated with DNase I and RNase A for 5 min, and then incubated with 10 $\mu$l of C217-coupled beads for 2 h at 4°C. After being washed in buffer with 0.05% Tween-20, the immunoprecipitate was subjected to Western blot analysis. For standard samples, different amounts of purified rVpr were added to 200 $\mu$l of control plasma. No detergents were added when the samples were incubated with the primary antibody, so that the IP-WB would detect only soluble Vpr, and not Vpr in viral particles.[10] The detection limit of the system was about 1 ng/ml (0.07 nM) (SI-2a).

Representative results from the IP-WB analysis of 14 plasma samples are shown in Fig. 1a. A definite signal of the 14-kDa protein was observed in patients N-09, 11, and 13 (Fig. 1a). By contrast, no peptides around 14 kDa were detected in more than 60 specimens from healthy volunteers (Fig. 1b). Because the IP-WB could selectively detect the 14-kDa peptide in the culture supernatant of cells containing an expression plasmid encoding *vpr* (SI-2b), we concluded that the 14-kDa peptide detected by the IP–WB was Vper. A comparison of the signal intensities of the detected bands and standard rVpr (Fig. 1a; 5, 2.5, and 1.25 $\mu$g/ml signals, and N-11) indicated that the serum Vpr concentration was about 0.7 nM.

During the analysis, we did not detect the Vpr signal in one patient (N-10; Table 1) who had 11,000 copies/ml of HIV-1 RNA (Fig. 1a, lower panel). To evaluate whether our system failed to detect Vpr mutants differing from the prototype NL4-3 (GenBank accession number M19921), we amplified DNA fragments from peripheral blood mononuclear cells covering the entire *vpr* gene. Then we determined its nucleotide sequence (Fig. 2a, and primers in SI-3). The deduced amino acid sequences are also shown in Fig. 2b. Interestingly, the *vpr* gene from patient N-10 had a four-nucleotide (TTAA) insertion at

Table 1. Clinical Data of Patients Subjected to Analysis and Results of the IP-WB

| | | | | | | Clinical data | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | White blood cells (/mm$^3$) | | | | |
| *Case number* | *Sex* | *Age* | *Causes of infection* | *Conditions* | *Treatment status*[a] | *Total number* | *Lymphocytes* | *CD4$^+$ T cells* | *HIV-1 RNA (copies/ml)* | *Vpr*[b] |
| N-01 | M | 39 | HO[c] | AIDS[d] | 2 | 8400 | 2612 | 771 | <50 | − |
| 02 | M | 41 | HO | AIDS | 2 | 6800 | 2584 | 346 | <50 | − |
| 03 | M | 59 | HE[c] | AIDS | 2 | 4700 | 2444 | 381 | 260 | − |
| 04 | F | 32 | HE | AC[d] | 3 | 6300 | 1890 | 302 | 4,400 | − |
| 05 | M | 38 | HO | AIDS | 2 | 8600 | 2417 | 585 | <50 | − |
| 06 | M | 35 | HO | AC | 1 | 4900 | 1274 | 116 | 220,000 | +++ |
| 07 | M | 45 | BL[c] | AIDS | 2 | 2600 | 546 | 38 | 73,000 | ++ |
| 08 | M | 58 | HE | AIDS | 2 | 6800 | 1632 | 366 | <50 | − |
| 09 | M | 29 | BL | AC | 1 | 3000 | 1056 | 266 | 17,000 | + |
| 10 | M | 23 | HO | AC | 1 | 5200 | 1300 | 230 | 11,000 | − |
| 11 | F | 37 | HE | AC | 1 | 4600 | 1150 | 222 | 500,000 | +++ |
| 12 | F | 40 | HE | AC | 1 | 6600 | 1584 | 598 | 98 | − |
| 13 | M | 42 | BL | AIDS | 2 | 3100 | 1054 | 110 | 70,000 | ++ |
| 14 | M | 23 | HO | AC | 1 | 5800 | 2656 | 553 | 71,000 | ++ |

[a]Group 1, no therapy; group 2, under medication; group 3, posttherapy.

[b]Based on results of the IP-WB, patients are divided into four groups; Vpr-negative (−) and Vpr-positive with less than 1 ng/ml (+), with 1–5 ng/ml (++), and with more than 5 ng/ml (+++).

[c]HO, homosexual; HE, heterosexual; BL, blood products.

[d]AIDS, acquired immunodeficiency syndrome; AC, asymptomatic carrier.

nucleotide 81, designated "clone 10," which generates a frameshift mutation within the inserted sequence (shown by the box in Fig. 2a). However, because this patient had no deletion in the 3′ region of the *vpr* gene, it was possible to clone the gene. Repeated sequence analyses of several clones of the amplified *vpr* DNA indicated that clone 10 was the major *vpr* in this patient (Table 2). The negative results of the IP-WB analysis for patient N-10 were therefore due to truncation of the C-terminal region.

Additional sequence analysis revealed that "clone N (Nara)," which differs by four amino acids from the prototype NL4-3 (Fig. 2b), was frequently observed in the analyzed patients (patients N-04, 05, 08, 09, 11, and 12). Interestingly, although patient N-09 had clone N as a major variant—all seven clones sequenced from the PCR products were identified as clone N (see Table 2)—the IP-WB analysis (Fig. 1a, lower panel) detected a positive Vpr signal in patient N-09. This suggests that C217 antibody, which was used as the first antibody in immunoprecipitation, reacts with the protein encoded by clone N, even though its C-terminal region differs from the prototype NL4-3 clone by two amino acids (Fig. 2b).

Next, we examined the possible correlation of Vpr and clinical manifestations. An analysis of 14 patients suggested a positive link between Vpr and viral titers (data not shown). To examine this possibility, we analyzed an additional 38 stocked samples using IP-WB. We detected Vpr in 14 samples. A representative result of the second analysis is shown in Fig. 1c. Positive Vpr signals were detected in patients T-166, 167, and 175. Then we examined the relationship between Vpr and RNA copy number in total 52 samples. As shown in Fig. 3a, we found a positive correlation between the detection of Vpr and RNA copy number ($p < 0.03$). In contrast, we did not detect a positive relationship between Vpr and the numbers of $CD4^+$ T cells or total white blood cells. The distribution of Vpr-positive patients based on the concentration of Vpr implied that the high amount of Vpr is observed in patients with high HIV-1 RNA copy numbers (Fig. 3b).

In the current work, we successfully identified Vpr in 20 samples from 52 HIV-1-positive patients. A comparison of the signals obtained with standard rVpr revealed that the Vpr concentration was ~0.7 nM. Levy *et al.*[9] suggested that Vpr is present in patient plasma, with rVpr activating viral reproduction when added to the culture medium of latently infected cells. In addition, Muthumani *et al.* proposed that exogenous rVpr has various activities, such as inducing T cell apoptosis,[11] inhibiting macrophage function,[12] and suppressing NF-$\kappa$B signaling.[13] However, these experiments did not consider the actual amount of Vpr present in the plasma samples. Our result is the first demonstration of Vpr in HIV-1-positive patients, and provides a rationale for the dose of rVpr suitable for *in vitro* experiments.

We observed a positive correlation between the detection of Vpr and HIV-1 RNA copy number ($p < 0.03$) (Fig. 3a). It has been reported that the exogenous Vpr induces viral production from latently infected cells, implying that Vpr is involved in viral reproduction *in vivo*. An important question still to be answered is how the Vpr titer changes in the context of viral replication during the clinical course of the disease. It is important to clarify whether Vpr functions as an initial trigger of viral expansion *in vivo*.

We did not detect a link between Vpr and the numbers of $CD4^+$ T cells. Recently, it was determined that WT-Vpr and its variant R77Q act differently in modifying the clinical features of HIV-1-positive patients. Based on several reports, it has been proposed that R77Q is a candidate marker for long-term nonprogression (LTNP),[14–16] although this is still controversial.[17,18] In this study, we observed that the main Vpr variants of patients N-04, 09, and 10 were R77Q or C-terminally truncated. However, we did not recognize these patients as candidates for LTNP (clinical observation by M. Konishi). The involvement of WT-Vpr and R77Q in patients is rationalized by *in vitro* experiments showing that rVpr induces the apoptosis of $CD4^+$ T cells,[11,12,14,15] whereas R77Q has less potent apoptosis activity than WT-Vpr.[15] It is important to note that the *in vitro* studies of the differential activities of exogenous WT-Vpr and R77Q used tremendous amounts of the proteins, and a difference in activity was observed only when 1.5–2.0 $\mu$M of the peptides was used.[15] As shown here, the concentration of Vpr in patient plasma was a maximum of 1.0 nM, and it is crucial to compare the functional difference of these molecules at a concentration comparable to that observed *in vivo*. Careful studies are required to address this matter.

## SUPPLEMENTARY INFORMATION

**SI-1.** Purification of rVpr and measurement using ELISA. (**a**) Expression and purification profiles of rVpr. Vpr was expressed as a fusion protein with GST and purified in a glutathione column. Lane 1, marker; lane 2, initial lysate; lane 3, flow-through sample eluted from the glutathione column; lane 4, eluate from rVpr after treatment with precision protease; and lane 5, eluate from an affinity column containing a monoclonal antibody against Vpr (8D1). The arrowhead and arrow indicate the position of GST-Vpr and purified rVpr, respectively. Proteins were stained with Coomassie brilliant blue solution. (**b**) ELISA verion-1 for measuring rVpr. Synthesized full-length Vpr was used to make a standard curve. To the Vpr-ELISA were added 10 ng/ml each of GAPDH, HIV-1 integrase, and SARS-CoV Spike protein, which were expressed as a (His)-tagged protein, and purified using Ni-beads. Note that none of the samples gave cross-signals with Vpr. The amount of rVpr was assessed using the absorbance at OD450 nm, as shown with the dotted line.

**SI-2.** Detection of Vpr by the IP-WB. (**a**) Sensitivity of the system. The IP-WB analysis was conducted using C217 for IP and 8D1 for WB. To determine the sensitivity of the system, 10, 5, 2.5, and 1.25 ng of purified rVpr were added to 200 $\mu$l of plasma from a healthy human just before the IP-WB analysis. The signals obtained using IP-WB (upper panel) and the input rVpr (lower panel) detected by 8D1 are shown. (**b**) Detection of Vpr in a culture supernatant. Culture supernatants (sup.) of 293FS cells (Invitrogen) transfected with pcDNA3.1 (center lane, "Vec") or pcDNA3.1-*vpr* (right lane, "Vpr") were collected on day 6 after transfection, and the IP-WB analysis was carried out. The rVpr (400 pg/lane) was included in the same blot as a positive control of WB (left lane).

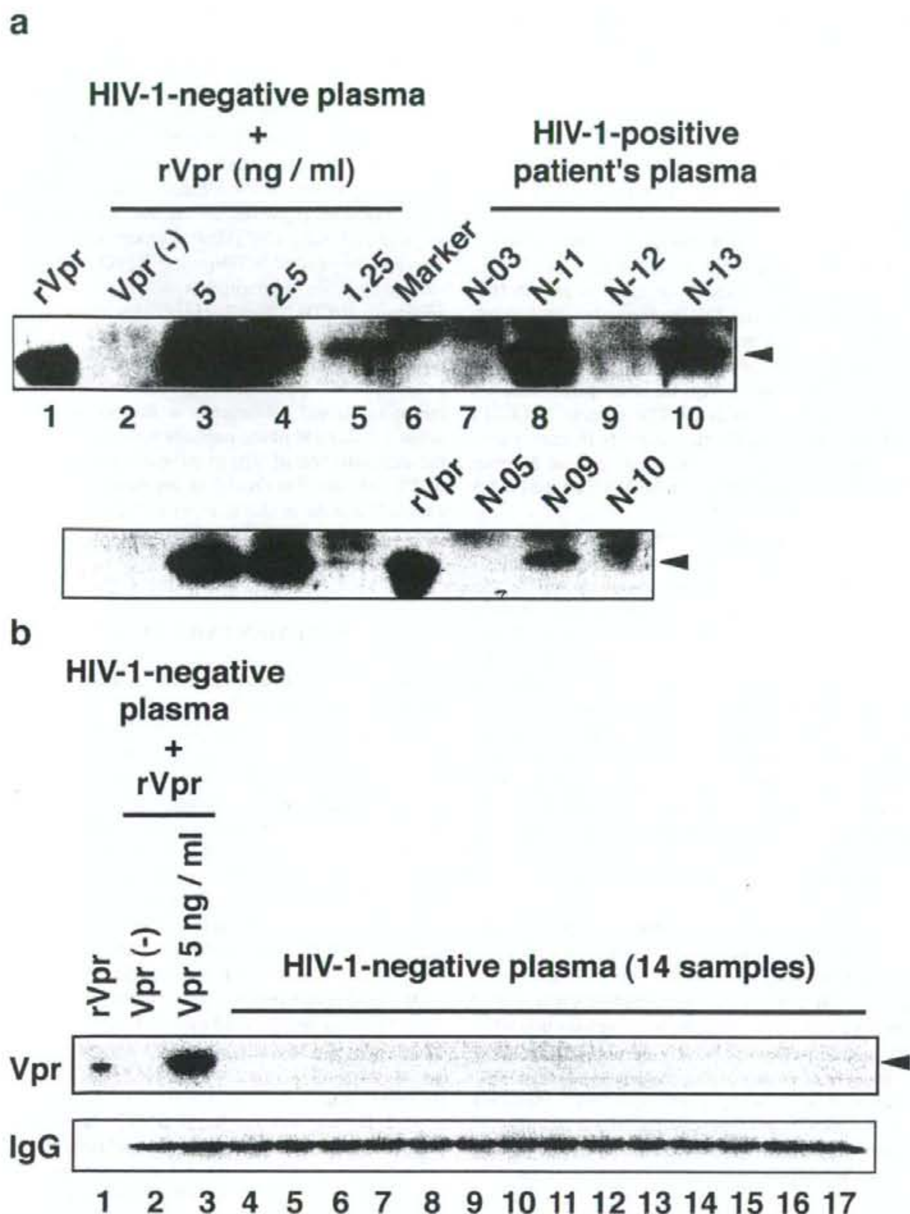


**FIG. 1.** Detection of Vpr in sera of HIV-1-positive patients. (**a**) Presence of the 14-kDa Vpr protein in HIV-1-positive patients. To semiquantify the Vpr concentration in patient samples, 5, 2.5, and 1.25 ng of standard rVpr (lanes 2–5), which had been measured using ELISA version-1 (see supplementary information 1b; SI-1b), were included. As a positive control for the WB analysis, 1 ng of rVpr (lane 1) was also included. Signals of HIV-1-positive plasma (lanes 7–10) and a molecular marker (lane 6) are shown. (**b**) Representative results of the IP-WB analysis of healthy volunteers. The IP-WB analysis was performed on more than 60 samples from healthy volunteers, and representative results from 14 cases (lanes 4–17) are shown. Note that no signals were detected around 14 kDa. The results for input rVpr (lane 1), no rVpr (lane 2), or 5 ng Vpr (lane 3) added to normal plasma are shown. IgG signals recovered after IP are also shown (lower panel). (**c**) Detection of the 14-kDa Vpr protein in HIV-1-positive patients in the second group. Also in this analysis, 5, 2.5, and 1.25 ng of standard rVpr (lanes 2–5) were included to assess the concentration of Vpr in patient plasma samples.

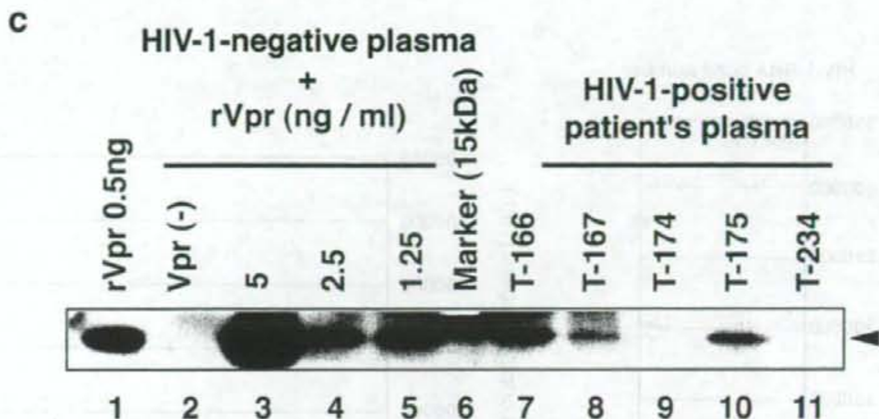


FIG. 1. *(Continued).*

**SI-3.** Cloning and sequence analysis of *vpr.* DNA covering *vpr* was amplified from the genomic DNA of peripheral blood cells using nested PCR. The primers used were Vpr1F (nt 4713–4733, 5′-GACCCTGACCTAGCAGACCA-3′) and Vpr1R (nt 5298–5318, 5′-CAAACTTGGCAATGAAAGCA-3′) for the first PCR. For the second PCR, Vpr2F (nt. 4854–4875, 5′-CAGTACTTGGCACTAGCAGCA-3′) and Vpr2R (nt 5243–5263, 5′-TAGGCTGACTTCCTGGATGC-3′) were used (GenBank accession number M19921). The first and second rounds of PCR were performed for 30 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min and for 95°C for 30 sec, 64°C for 30 sec, and 72°C for 45 sec, re-

**a**

```
NL4-3      1 ATGGAACAAG CCCCAGAAGA CCAAGGGCCA CAGAGGGAGC CATACAATGA ATGGACACTA
Clone-10   1 ---------- ---------- ---------- ---------- ---------- ----------

          61 GAGCTTTTAG AGGAACTTAA     GAGTGA AGCTGTTAGA CATTTTCCTA GGATATGGCT
          61 ---------- ---------- TTAA------ ---------- ---------- ----------

         117 CCATAACTTA GGACAACATA TCTATGAAAC TTACGGGGAT ACTTGGGCAG GAGTGGAAGC
         121 ---------- ---------- ---------- ---------- ---------- ----------

         177 CATAATAAGA ATTCTGCAAC AACTGCTGTT TATCCATTTC AGAATTGGGT GTCGACATAG
         181 ---------- ---------- ---------- ---------- ---------- ----------

         237 CAGAATAGGC GTTACTCGAC AGAGGAGAGC AAGAAATGGA GCCAGTAGAT CCTAG 291nt.
         241 ---------- ---------- ---------- ---------- ---------- ----- 295nt.
```

**b**

```
NL4-3     MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS
Clone-10  ••••••••••••••••••••••••••N*
Clone-N   ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••Q•••••II••••••••••••
```

FIG. 2. Sequence analysis of *vprs* and the deduced amino acids of Vpr variants in HIV-1-positive patients. The *vpr* gene was amplified and analyzed, as described in SI-3. (**a**) Nucleotide sequence of clone 10. The nucleotide sequence was compared with that of the prototype NL4-3. Clone 10 has a four-base insertion at nucleotide 81, generating a stop codon within the insert (indicated by the box). Nucleotides that are the same as those in NL4-3 are marked with small bars. (**b**) Amino acid sequences of Vpr variants found in the patients. The amino acids deduced from the obtained sequences and the NL4-3 clone are shown. As described in SI-3a, clone 10 was recognized as a major variant in patient N-10, while clone N was the major variant in patients N-04 and 09.

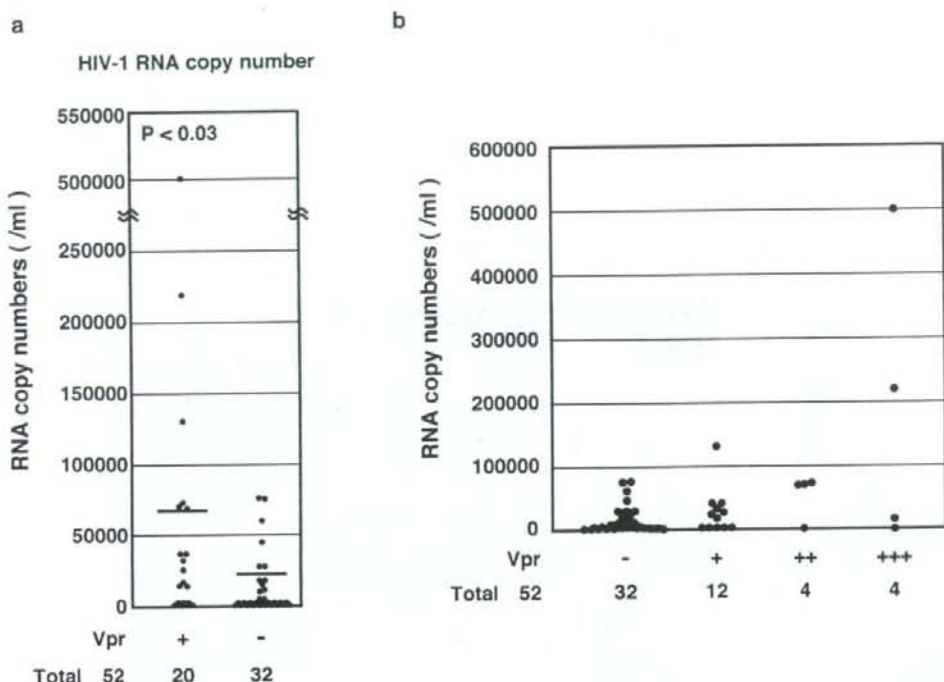


**FIG. 3.** Correlation between Vpr detection and clinical data. The analyzed cases were divided into Vpr-positive and Vpr-negative groups, and the statistical analysis was done using Student's *t*-test. (**a**). The relationships with the HIV-1 RNA copy number. The bars indicate the mean numbers in each group. The difference for HIV-RNA copy number with Vpr was statistically significant ($p < 0.03$). (**b**) Distribution of Vpr-positive patients according to the concentration of plasma Vpr. Based on the semi-quantitative analysis, patients were divided into four groups: Vpr-negative (−), Vpr-positive with less than 1 ng/ml (+), 1–5 ng/ml (++), and more than 5 ng/ml (+++). Each dot means a patient.

spectively. The PCR products were cloned into pZeroBlunt topo vector (Invitrogen, Carlsbad, CA). Several clones were sequenced for each PCR product.

**SI-4.** See Table 2.

TABLE 2. FREQUENCY OF *vpr* VARIANTS IN HIV-1 PATIENTS[a]

| | vpr *variants* | | |
|---|---|---|---|
| *Cases* | *NL4-3* | *Clone N* | *Clone 10* |
| N-03 | 7[b] | — | 9 |
| N-04 | — | 14 | — |
| N-05 | 2 | 2 | 3 |
| N-09 | — | 7 | — |
| N-10 | — | — | 5 |
| N-12 | 4 | 4 | — |

[a]PCR products amplified from patient genomic DNA were subcloned into the vector, and several clones were sequenced. The numbers in the table indicate the frequency of clones encountered in the sequence analyses. All 5 clones derived from patient N-10 were clone 10. In patients N-04 and 09, clone N was identified as the major variant; all 14 clones for patient N-04 and all 7 clones for patient N-09 were clone N. Patients N-03 and N-05 each had 2 *vpr* variants.

[b]Number of analyzed clones.

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INTERNAL MEDICINE

□ CASE REPORT □

# Fatal Cytomegalovirus-Associated Adrenal Insufficiency in an AIDS Patient Receiving Corticosteroid Therapy

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## Abstract

A 35-year-old homosexual man, who had already received sulfamethoxazole/trimethoprim and steroid therapy because of human immunodeficiency virus (HIV)-related *Pneumocystis jiroveci* pneumonia, was referred to our hospital. He was also diagnosed as having cytomegalovirus (CMV) co-infection, and started receiving intravenous gancyclovir for CMV infection on the 2nd day of admission into our hospital. He had to continue the steroid therapy because his respiratory condition did not improve. On the 10th hospitalization day, when 40 mg of prednisolone was administered, cardiopulmonary arrest suddenly occurred, and his laboratory data showed hyponatremia and hyperpotassemia. In spite of resuscitation, he died two days later. The postmortem examination revealed that he died of adrenal failure due to CMV infection. In general, CMV is thought to cause adrenalitis, but rarely leads to manifestations of adrenal insufficiency during the clinical course. It is important to be aware that grave adrenal failure due to CMV infection can develop even under steroid therapy.

**Key words:** AIDS, cytomegalovirus, adrenal insufficiency, corticosteroid

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## Introduction

Recent advances in antiretroviral therapies have dramatically decreased the mortality rate of acquired immunodeficiency syndrome (AIDS). Despite these advances, opportunistic infections still develop, particularly in the patients who already have severe immunosuppression following the diagnosis of HIV infection. Predilection of cytomegalovirus (CMV) to the adrenal gland has been recognized, and many autopsy studies have revealed CMV invasion to the adrenal gland in AIDS patients. However, clinical adrenal insufficiency rarely develops in HIV-infected patients. We herein report an AIDS patient who died of CMV-induced adrenal failure detected on autopsy examination.

## Case Report

A 35-year-old homosexual man had non-productive cough, fever, and exertional dyspnea since May 2004. He went to a neighborhood hospital 12 days before admission into our hospital. He was given azithromycin, but his symptoms did not improve, and he was admitted into the hospital 10 days before he was referred to our hospital because his chest radiography revealed a diffuse interstitial infiltrate. At the previous hospital, 1,000 mg of pazufloxacin was administered, but his respiratory condition became worse. Seven days before admission into our hospital, steroid pulse therapy (1 g/day) was administered for 3 days because of severe hypoxemia. His plasma β-D-glucan was 271 pg/ml 6 days before admission, and 12 tablets of sulfamethoxazole (4.8 g)/trimethoprim (960 mg) and 50 mg of itraconazole were administered 4 days before admission. He was referred to our hospital on June 25 because his serum HIV-1 antibody was positive (Fig. 1).

On admission, his blood pressure was 86/54 mmHg, and his respiratory rate was 30/min. Fine crackles were heard over his bilateral lower lung fields, and hepatomegaly was detected by palpation.

Laboratory data on admission showed anemia, hypoalbu-

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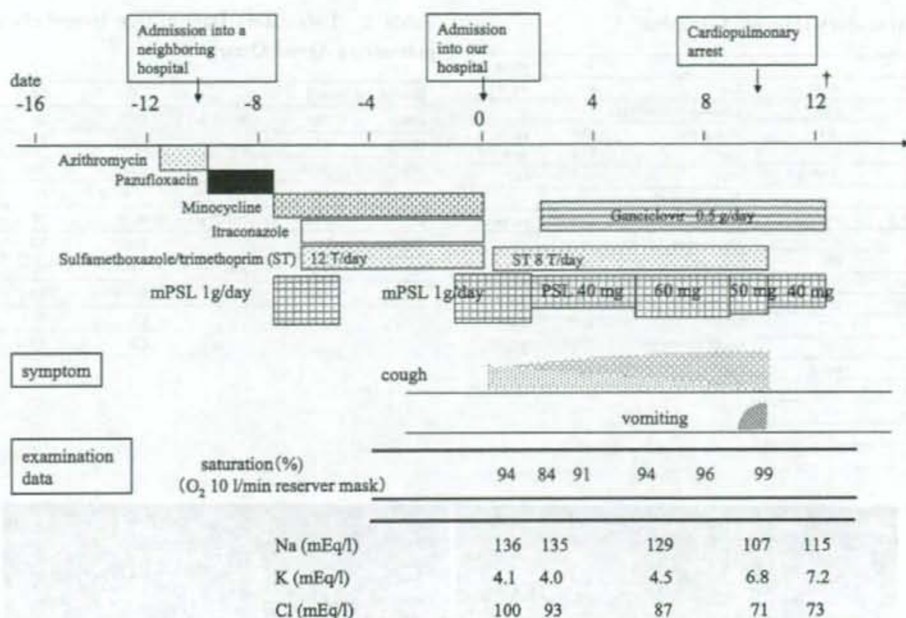


**Figure 1. The clinical course of this case.**

minemia, and high level of lactate dehydrogenase, C-reactive protein, and plasma β-D-glucan. The CD4-positive cell count was 13/μl, and the HIV-RNA loads were $1.1\times10^5$ copies/ml. The CMV antigenemia testing was positive (C7-HRP 60/150000 of WBC cells). Arterial blood gas analysis under 10 l/min oxygen inhalation (reserver mask) showed $PaO_2$ of 71 Torr and $PaCO_2$ of 31.3 Torr (Table 1). Chest radiography showed a diffuse interstitial infiltrate with ground glass appearance.

We diagnosed him as having pneumocystis pneumonia and CMV infection. The treatment for pneumocystis pneumonia with 8 tablets of sulfamethoxazole (3.2 g)/ trimethoprim (640 mg) and 1 g of methylprednisolone (mPSL) was continued. In addition, administration of intravenous gancyclovir for CMV infection was started on the 2 nd day. His percutaneous oxygen saturation gradually improved, we reduced the amount of steroids to 40 mg of prednisolone on the 3rd day. However, he continued to complain of cough and dyspnea, so we added prednisolone (60 mg) on the 5th day, tapered to 50 mg on the 6th day, and to 40 mg on the 10th day. He has also complained of vomiting since the 9th hospitalization day.

On the 10th day, he suddenly fell into cardiopulmonary arrest. The laboratory tests one hour before the cardiopulmonary arrest showed serum sodium level of 107 mEq/l, chloride level of 71 mEq/l, and serum potassium level of 6.8 mEq/l (Table 2). Despite resuscitation, he died on the 12th day of admission (Fig. 1). Autopsy examination was performed 12 hours later. The weight of the right and left adrenal glands was 15 g and 10 g, respectively. An extensive hemorrhagic necrosis was macroscopically observed in both adrenal glands (Fig. 2a). Microscopic examination revealed diffuse hemorrhage and necrosis with intranuclear inclusion bodies in the bilateral adrenal glands (Fig. 2b, c). These findings were compatible with CMV-induced adrenal insufficiency. Diffuse alveolar damage and pulmonary hemorrhage were noticed in the bilateral lungs. Intranuclear inclusion bodies and cysts of *Pneumocystis jiroveci* were found in the lesions.

## Discussion

The direct cause of death in this patient was considered to be mortal arrhythmia with hyperpotassemia due to CMV-associated adrenal insufficiency, which was diagnosed by his clinical features and post-mortem extermination. The differential diagnoses of electrolyte imbalance included acute renal failure, gastrointestinal losses, syndrome of inappropriate secretion of ADH (SIADH), and drug-induced. Acute renal failure was excluded because his serum creatinine level was in the normal range, and gastrointestinal losses were also excluded because there was no diarrhea. Agarwal et al reported that 36 of 103 patients (35%) with AIDS who were admitted for acute opportunistic infections had serum sodium of 130 mEq/l or less, and 23 patients had concomitant SIADH (1). Nevertheless, we could not deny SIADH because we checked neither ADH nor the urine electrolytes. Regarding the drug-induced electrolyte imbalance, Bevilacqua et al reported that a high dose of trimethoprim acted as an amiloride-like drug and induced a clinical state characterized by hyponatremia and hyperkalemia which is indistinguishable from hyporeninemic hypoaldosteronism (2). We

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**Table 1. Laboratory Data on Admission**

| 【Peripheral blood】 | | | BUN | 10 | mg/dl |
|---|---|---|---|---|---|
| RBC | 369 | $\times 10^4/\mu l$ | Cre | 0.6 | mg/dl |
| Hb | 11.0 | g/dl | 【Serology/Immunology】 | | |
| WBC | 5400 | $/\mu l$ | β-D-glucan | 69.6 | pg/ml |
| lym | 9.0 | % | CRP | 13.1 | mg/dl |
| PLT | 23.9 | $\times 10^4/\mu l$ | $CD4^+$ | 13 | $/\mu l$ |
| 【Biochemistry】 | | | 【Infection tests】 | | |
| TP | 5.7 | g/dl | HIV-RNA | 110000 | copies/ml |
| Alb | 2.6 | g/dl | Cryptococcus Ag | (-) | |
| GOT | 40 | IU/l | Aspergillus Ag | 0.1 | |
| GPT | 20 | IU/l | CMV Ag | (+) (60/150000) | |
| LDH | 780 | IU/l | 【Artery blood gas analysis (10 l/min mask)】 | | |
| ALP | 180 | IU/l | PO2 | 71 | Torr |
| γ-GTP | 29 | IU/l | PCO2 | 31.3 | Torr |
| Na | 136 | mEq/l | pH | 7.452 | |
| K | 4.1 | mEq/l | | | |
| Cl | 100 | mEq/l | | | |
| Ca | 8.1 | mg/dl | | | |

**Table 2. Laboratory Data at One Hour before the Cardiopulmonary Arrest Occurred**

| 【Peripheral blood】 | | | GOT | 24 | IU/l |
|---|---|---|---|---|---|
| RBC | 389 | $\times 10^4/\mu l$ | GPT | 30 | IU/l |
| Hb | 15.1 | g/dl | LDH | 374 | IU/l |
| WBC | 3800 | $/\mu l$ | ALP | 365 | IU/l |
| lym | 4.0 | % | γ-GTP | 53 | IU/l |
| PLT | 19.2 | $\times 10^4/\mu l$ | BUN | 28 | mg/dl |
| 【Biochemistry】 | | | Cre | 0.8 | mg/dl |
| TP | 7.8 | g/dl | Na | 107 | mEq/l |
| Alb | 4.0 | g/dl | K | 6.8 | mEq/l |
| | | | Cl | 71 | mEq/l |
| | | | Ca | 9.3 | mg/dl |

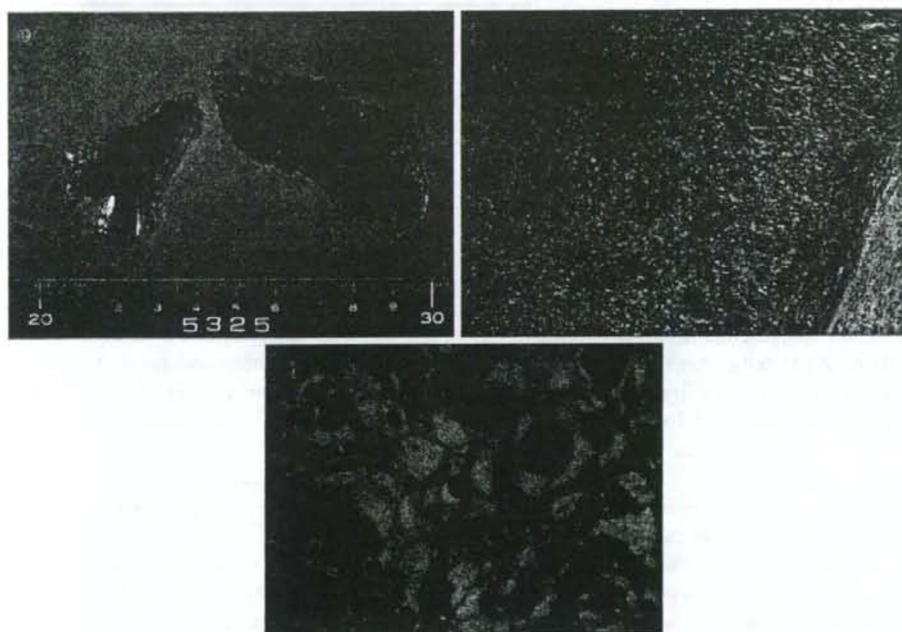


**Figure 2. Autopsy examination of the adrenal glands revealed an extensive hemorrhage and necrosis with intranuclear inclusion bodies. (HE stain)**

could not deny the influence of the drug-induced electrolyte imbalance, either.

CMV infection is a common opportunistic infection among the patients with advanced HIV disease. CMV infection can take many forms, including retinitis, colitis, esophagitis, encephalomyelitis, and other syndromes. Adrenal infection by CMV is a common autopsy finding in patients with AIDS (3). Pulakhandam *et al* reported that the incidence of adrenal involvement in 37 AIDS patients with CMV infection was 84% in their autopsy study (4). Grinspoon *et al* detected CMV in the adrenal glands of 33% to 88% of patients who died of AIDS (5). On the other hand, adrenal involvement by other pathogens is relatively rare (6). However, the clinical features of acute adrenal insufficiency are rare in AIDS patients with CMV infection. To our knowledge, there is the only one case report on CMV-induced adrenal insufficiency in a Japanese patient with AIDS (7). This is because glandular destruction does not typically exceed more than 50% of the adrenal tissue in most cases with CMV-induced adrenalitis (8). Because extensive adrenal hemorrhagic necrosis associated with CMV was observed in our post-mortem extermination, our patient was considered to have acute adrenal insufficiency.

The grave adrenal insufficiency was probably caused by

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the delay of therapy for CMV and the treatment of pneumocystis pneumonia with steroid in our case. Adrenal insufficiency rarely develops in patients receiving supraphysiological doses of steroid for concomitant illness (9, 10). Razzaq *et al* first reported the development of CMV-induced adrenal failure in a patient with AIDS while receiving corticosteroid therapy. They reported that their patient had no manifestations related to his adrenal insufficiency which only became apparent on routine blood testing (11). They noticed a decrease in the serum sodium level, and doubted the occurrence of adrenal insufficiency. In the present case, vomiting was the only symptom that might have been related to adrenal insufficiency, and it occurred one day before cardiopulmonary arrest. Methylprednisolone and prednisolone, which were administered to our patient, have a weaker mineralocorticoid effect than glucocorticoid effect. Therefore, various manifestations associated with the glucocorticoid effect became unclear, and we did not notice his adrenal insufficiency until his serum electrolytes became deranged. The differential diagnoses of adrenal insufficiency in immunocompromised patients include viral, fungal, and mycobacterial diseases (12). Nonetheless, in our autopsy study, we found neither viral, fungal, nor mycobacterial infection except for CMV and *pneumocystis jirovecii* infection.

Some reports state that CMV-induced adrenal insufficiency improved with early appropriate treatment (6, 7). The electrolyte imbalance, which suggested the adrenal insufficiency, began on the 6th day of admission in our hospital. If we noticed this abnormality early, we could have saved his life. So, it is very important to pay careful attention to the manifestations of adrenal insufficiency in AIDS patients with CMV infection while receiving steroid therapy.

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# A CASE OF GYNECOMASTIA ASSOCIATED WITH EFAVIRENZ

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*Abstract* : We herein report on an HIV-infected patient with gynecomastia caused by efavirenz (EFV). A 57-year-old man was diagnosed with HIV-1 infection in 1994. He started therapy with zidovudine / lamivudine, didanosine, and EFV in December 2002. He felt painful breast hypertrophy after 5 months. There were firm, tender, unattached masses under the bilateral nipples, and ultrasonography showed benign enlargement of the breast without adipomastia. The blood levels of thyroid hormones, testosterone, estradiol, prolactin, and cortisol were normal. Therefore, we diagnosed him as having EFV-induced gynecomastia because of medication history and examinations. Although EFV was not stopped, his gynecomastia gradually improved.

**Key words** : human immunodeficiency virus infection, gynecomastia, efavirenz, adverse effect

## INTRODUCTION

The prognosis of human immunodeficiency virus (HIV) infection has dramatically improved in the era of highly active antiretroviral therapy (HAART). However, HAART has been associated with the development of numerous acute and long-term adverse effects. Some morphologic changes are often observed as adverse effects of HAART[1]. There have been recent reports describing gynecomastia in HIV-infected men treated with HAART[2-5]. We describe herein an HIV-infected man with gynecomastia that developed after receiving efavirenz (EFV).

## CASE REPORT

A 57-year-old homosexual man was diagnosed with HIV-1 infection at a public health office in December 1994. He commenced the antiretroviral therapy in October 1995, but often needed to change the regimen of antiretroviral therapy because of insufficient effects or adverse reactions. He received zidovudine (AZT) between October 1995 and July 1996, AZT and didanosine (ddI) between July 1996 and July 1997, AZT and lamivudine (3TC) between July 1997 and October 1997, AZT, 3TC and nelfinavir (NFV) between October 1997 and April 1998, stavudine (d4T), ddI and NFV between April 1998 and December 1998, d4T, ddI and indinavir (IDV) between December 1998 and August 2001, and d4T, ddI, IDV and ritonavir (RTV) between August 2001 and December 2002. He started the therapy with AZT/3TC, ddI and EFV in December 2002. EFV was the only agent administered for the first time. He had not received any other medications. His CD4 positive cell count was kept over 500/$\mu$l, and his

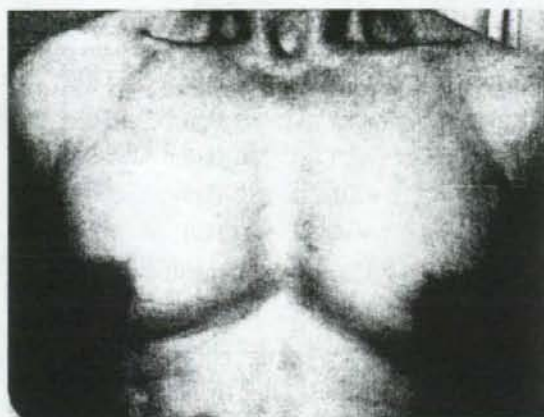

Fig. 1. Gynecomastia in this patient. The firm, tender, unattached masses were observed under his bilateral nipples

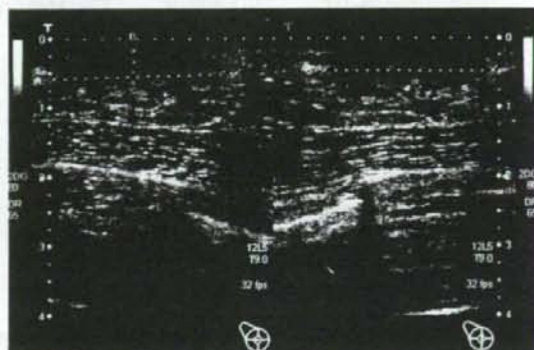

Fig. 2. Ultrasonography of the bilateral breasts showed ill-defined, heterogenous, echo-poor areas of nodularity deep to the nipple

plasma viral load was the limit of detection (<50 copies/ml). He felt painful breast hypertrophy at the end of May 2003. His height was 150.0 cm, and his weight was 47.6 kg. His blood pressure was 118/70 mmHg. There were firm, tender, unattached masses under the bilateral nipples (Fig. 1). His testicles were normal, and he showed no manifestations of lipodystrophy syndrome. Laboratory data showed a red blood cell count of $340 \times 10^4/\mu l$, serum aspartate aminotransferase of 36 IU/l, lactate dehydrogenase of 232 IU/l, $\gamma$-glutamyl transferase of 129 IU/l, creatinine of 1.2 mg/dl, triglycerides of 151 mg/dl, and total cholesterol of 268 mg/dl. The CD4 positive cell count was $591/\mu l$, and the HIV-RNA load remained undetectable. The blood levels of thyroid hormones, testosterone, estradiol,

Table 1. Table 1. Laboratory data

| [Peripheral blood] | | [Blood chemistry] | | | |
|---|---|---|---|---|---|
| RBC | 340×10⁴ /μl | AST | 36 IU/l | CD4⁺ | 591 /μl |
| Hb | 13.8 g/dl | ALT | 36 IU/l | CD8⁺ | 929 /μl |
| Ht | 39.0 | LDH | 232 IU/l | [Virology] | |
| WBC | 5400 /μl | γ-GTP | 129 IU/l | HBsAg | − |
| meta | 1 % | UA | 6.1 mg/dl | HCVAb | − |
| st | 1 % | BUN | 20 mg/dl | HIV-RNA | <50 copies/ml |
| seg | 60 % | Cre | 1.2 mg/dl | [Endocrine] | |
| ba | 1 % | TG | 151 mg/dl | TSH | 1.97 μU/ml |
| lym | 31 % | T.cho | 268 mg/dl | T3 | 1.03 ng/ml |
| mo | 6 % | Glu | 99 mg/dl | T4 | 6.4 μg/dl |
| Plt | 24.6×10⁴ /μl | [Serology/immunology] | | Cortisol | 11.5 μg/dl |
| | | CRP | 0.1 mg/dl | PRL | 8.6 ng/ml |
| | | | | Estoradiol | 36.5 pg/ml |
| | | | | Teststerone | 5.9 ng/ml |

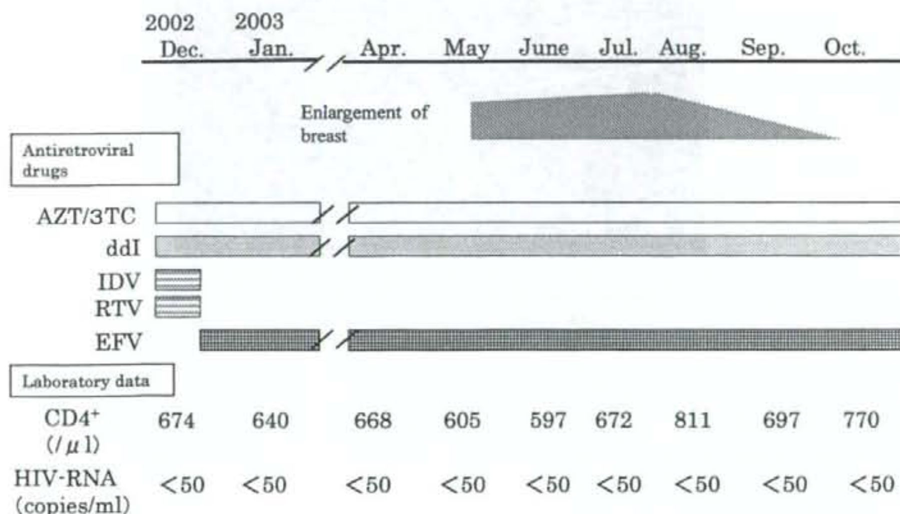


Fig. 3. Clinical course of this patient. AZT/3TC : zidovudine/lamivudine, ddI: didanosine, IDV: indinavir, RTV: ritonavir, EFV: efavirenz, CD4+: CD4 positive cell count.

prolactin and cortisol were normal (Table 1). Ultrasonography showed benign enlargement of the breast without adipomastia (Fig. 2).

We suspected gynecomastia associated with EFV because of the medication history and the results of examinations. We did not change the regimen of HAART because HAART was effective and his gynecomastia was mild. However, the size of his breasts regressed and they became no longer tender or painful after 5 months (Fig. 3).

## DISCUSSION

Gynecomastia is defined as the development of prominent breast tissue in male. True gynecomastia is a condition in which there is an enlargement of the male breast due to an increase in the ductal tissue and periductal stroma[6]. On the other hand, lipomastia (pseudogynecomastia) is characterized by increased amounts of adipose tissue. Gynecomastia is recognized in 36–65% of normal men[7]. It is a normal physiologic al finding in 3 groups of males : newborns in whom it is transient due to exposure to maternal estrogens, adolescents who experience a transient gynecomastia in association with puberty, and elders in whom the androgenic activity decreases with aging. Gynecomastia has several etiologies, such as liver disease, hyperthyroidism, neoplasms of the testicles, pituitary tumors, adrenal tumors, and the use of drugs. Drugs can cause gynecomastia in several ways : acting as direct estrogen receptor agonists, increasing the estrogenic activity, enhancing the testicular estrogen secretion, interfering with the production of testosterone, or blocking the binding of testosterone to its receptor[8].

Our patient was not receiving other medications that could have caused gynecomastia, and this disorder appeared a few months after he began the HAART regimen including EFV that was the only agent received for the first time. He had no evidence of lipodystrophy syndrome. There were no biological or hormonal abnormalities that could explain gynecomastia in this case. These findings suggest that EFV can produce gynecomastia without lipodystrophy syndrome.

Gynecomastia has rarely been reported in HIV-infected men. However, gynecomastia has been increasingly reported in HIV-infected men taking several antiretroviral drugs since the introduction of HAART. At first, nucleoside reverse transcriptase inhibitors or protease inhibitors probably played a causal role[2-5]. Recently, there have been some reports on gynecomastia in HIV-infected men that was associated with EFV belonging to non-nucleoside reverse transcriptase inhibitors[9-11]. Therefore, we have to consider that gynecomastia may occur in HIV-infected men treated with several different classes of antiretroviral drugs.

The mechanisms underlying the development of EFV-associated gynecomastia remain unclear. Different hypotheses explaining this pathogenesis have been described in several reports. Qazi et al suggested that gynecomastia might represent a manifestation of immune restoration disease, because gynecomastia developed only in HIV-infected men who had excellent response to HAART[12]. The improvement of the helper T-cell cytokines response may influence the growth of breast tissue after commencement of an effective HAART. Besides, cytochrome P-450 inhibition induced by antiretroviral agents can elevate the

estrogen-androgen ratio. EFV was found to increase by 37% in the area under the curve (AUC) of coadministered ethynil estradiol, due to inhibition of cytochrome P-450[13]. Furthermore, EFV may have estradiol-like effects in the human body, triggering the growth of breast tissue. Sinicco et al found that blood samples from HIV-infected patients receiving EFV had unusually high levels of the female estradiol as measured by enzyme-linked immunosorbent assay (ELISA) method. Thus, EFV may bind to parts of the ELISA test that normally detect estradiol[14].

In conclusion, we report herein a case of gynecomastia associated with EFV. Our experience could explain the possible occurrence of gynecomastia in HIV-infected men receiving various antiretroviral drugs. Therefore, we should pay attention to gynecomastia in the long-term follow-up of HIV-infected men treated with HAART.

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