14. Huang KH, Goedhals D, Carlson JM, Brockman MA, Mishra S, Brumme ZL, *et al.* **Progression to AIDS in South Africa is associated with both reverting and compensatory viral mutations.** *PLoS One* 2011; **6**:e19018.
15. Rousseau CM, Daniels MG, Carlson JM, Kadie C, Crawford H, Prendergast A, *et al.* **HLA class I-driven evolution of human immunodeficiency virus type 1 subtype c proteome: immune escape and viral load.** *J Virol* 2008; **82**:6434–6446.
16. Avila-Rios S, Ormsby CE, Carlson JM, Valenzuela-Ponce H, Blanco-Heredia J, Garrido-Rodriguez D, *et al.* **Unique features of HLA-mediated HIV evolution in a Mexican cohort: a comparative study.** *Retrovirology* 2009; **6**:72.
17. Carlson JM, Listgarten J, Pfeifer N, Tan V, Kadie C, Walker BD, *et al.* **Widespread impact of HLA restriction on immune control and escape pathways of HIV-1.** *J Virol* 2012; **86**:5230–5243.
18. Carlson JM, Schaefer M, Monaco DC, Batorsky R, Claiborne DT, Prince J, *et al.* **HIV transmission. Selection bias at the heterosexual HIV-1 transmission bottleneck.** *Science* 2014; **345**: 1254031.
19. Chikata T, Carlson JM, Tamura Y, Borghan MA, Naruto T, Hashimoto M, *et al.* **Host-specific adaptation of HIV-1 subtype B in the Japanese population.** *J Virol* 2014; **88**:4764–4775.
20. Gesprasert G, Wichukchinda N, Mori M, Shiino T, Auwanit W, Sriwanthana B, *et al.* **HLA-associated immune pressure on Gag protein in CRF01_AE-infected individuals and its association with plasma viral load.** *PLoS One* 2010; **5**:e11179.
21. Dong T, Zhang Y, Xu KY, Yan H, James I, Peng Y, *et al.* **Extensive HLA-driven viral diversity following a narrow-source HIV-1 outbreak in rural China.** *Blood* 2011; **118**:98–106.
22. Buonaguro L, Tornesello ML, Buonaguro FM. **Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications.** *J Virol* 2007; **81**:10209–10219.
23. Lau KA, Wang B, Saksena NK. **Emerging trends of HIV epidemiology in Asia.** *AIDS Rev* 2007; **9**:218–229.
24. Rodrigo C, Rajapakse S. **Current Status of HIV/AIDS in South Asia.** *J Glob Infect Dis* 2009; **1**:93–101.
25. Carlson JM, Brumme CJ, Martin E, Listgarten J, Brockman MA, Le AQ, *et al.* **Correlates of protective cellular immunity revealed by analysis of population-level immune escape pathways in HIV-1.** *J Virol* 2012; **86**:13202–13216.
26. Carlson JM, Brumme ZL, Rousseau CM, Brumme CJ, Matthews P, Kadie C, *et al.* **Phylogenetic dependency networks: inferring patterns of CTL escape and codon covariation in HIV-1 Gag.** *PLoS Comput Biol* 2008; **4**:e1000225.
27. Brumme ZL, Poon AFY, Carlson JM, Walker BD. **Identifying HLA-Associated Polymorphisms in HIV-1.** In: Yusim K, Korber B, Brander C, Barouch D, de Boer R, Haynes BF,*et al.* editors. *HIV molecular immunology 2010* Los Alamos: Theoretical Biology and Biophysics Group, Los Alamos National Laboratory; 2010. pp. 3–8.
28. Storey JD, Tibshirani R. **Statistical significance for genomewide studies.** *Proc Natl Acad Sci U S A* 2003; **100**:9440–9445.
29. Bhattacharya T, Daniels M, Heckerman D, Foley B, Frahm N, Kadie C, *et al.* **Founder effects in the assessment of HIV polymorphisms and HLA allele associations.** *Science* 2007; **315**:1583–1586.
30. Brumme ZL, Brumme CJ, Heckerman D, Korber BT, Daniels M, Carlson J, *et al.* **Evidence of differential HLA class I-mediated viral evolution in functional and accessory/regulatory genes of HIV-1.** *PLoS Pathog* 2007; **3**:e94.
31. Matthews PC, Prendergast A, Leslie A, Crawford H, Payne R, Rousseau C, *et al.* **Central role of reverting mutations in HLA associations with human immunodeficiency virus set point.** *J Virol* 2008; **82**:8548–8559.
32. Rolland M, Carlson JM, Manocheewa S, Swain JV, Lanxon-Cookson E, Deng W, *et al.* **Amino-acid co-variation in HIV-1 Gag subtype C: HLA-mediated selection pressure and compensatory dynamics.** *PLoS One* 2010; **5**:e12463.
33. Wang YE, Li B, Carlson JM, Streeck H, Gladden AD, Goodman R, *et al.* **Protective HLA class I alleles that restrict acute-phase CD8+ T-cell responses are associated with viral escape mutations located in highly conserved regions of human immunodeficiency virus type 1.** *J Virol* 2009; **83**:1845–1855.
34. John M, Heckerman D, James I, Park LP, Carlson JM, Chopra A, *et al.* **Adaptive interactions between HLA and HIV-1: highly divergent selection imposed by HLA class I molecules with common supertype motifs.** *J Immunol* 2010; **184**:4368–4377.
35. Kiepiela P, Leslie AJ, Honeyborne I, Ramduth D, Thobakgale C, Chetty S, *et al.* **Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA.** *Nature* 2004; **432**:769–775.
36. Carlson JM, Le AQ, Shahid A, Brumme ZL. **HIV-1 adaptation to HLA: a window into virus-host immune interactions.** *Trends Microbiol* 2015; **23**:212–224.
37. Watanabe K, Murakoshi H, Tamura Y, Koyanagi M, Chikata T, Gatanaga H, *et al.* **Identification of cross-clade CTL epitopes in HIV-1 clade A/E-infected individuals by using the clade B overlapping peptides.** *Microbes Infect* 2013; **15**:874–886.
38. Leslie A, Kavanagh D, Honeyborne I, Pfafferott K, Edwards C, Pillay T, *et al.* **Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA.** *J Exp Med* 2005; **201**:891–902.
39. Brumme ZL, Brumme CJ, Carlson J, Streeck H, John M, Eichbaum Q, *et al.* **Marked epitope- and allele-specific differences in rates of mutation in human immunodeficiency type 1 (HIV-1) Gag, Pol, and Nef cytotoxic T-lymphocyte epitopes in acute/early HIV-1 infection.** *J Virol* 2008; **82**:9216–9227.
40. Prince JL, Claiborne DT, Carlson JM, Schaefer M, Yu T, Lahki S, *et al.* **Role of transmitted Gag CTL polymorphisms in defining replicative capacity and early HIV-1 pathogenesis.** *PLoS Pathog* 2012; **8**:e1003041.
41. Brumme ZL, Tao I, Szeto S, Brumme CJ, Carlson JM, Chan D, *et al.* **Human leukocyte antigen-specific polymorphisms in HIV-1 Gag and their association with viral load in chronic untreated infection.** *AIDS* 2008; **22**:1277–1286.
42. Norstrom MM, Buggert M, Tauriainen J, Hartogensis W, Prosperi MC, Wallet MA, *et al.* **Combination of immune and viral factors distinguishes low-risk versus high-risk HIV-1 disease progression in HLA-B*5701 subjects.** *J Virol* 2012; **86**:9802–9816.
43. Chopera DR, Woodman Z, Mlisana K, Mlotshwa M, Martin DP, Seoighe C, *et al.* **Transmission of HIV-1 CTL escape variants provides HLA-mismatched recipients with a survival advantage.** *PLoS Pathog* 2008; **4**:e1000033.
44. Gijsbers EF, Feenstra KA, van Nuenen AC, Navis M, Heringa J, Schuitemaker H, *et al.* **HIV-1 replication fitness of HLA-B*57/58:01 CTL escape variants is restored by the accumulation of compensatory mutations in gag.** *PLoS One* 2013; **8**:e81235.
45. Leslie AJ, Pfafferott KJ, Chetty P, Draenert R, Addo MM, Feeney M, *et al.* **HIV evolution: CTL escape mutation and reversion after transmission.** *Nat Med* 2004; **10**:282–289.
46. Goepfert PA, Lumm W, Farmer P, Matthews P, Prendergast A, Carlson JM, *et al.* **Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients.** *J Exp Med* 2008; **205**:1009–1017.
47. Martinez-Picado J, Prado JG, Fry EE, Pfafferott K, Leslie A, Chetty S, *et al.* **Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1.** *J Virol* 2006; **80**:3617–3623.
48. Navis M, Schellens I, van Baarle D, Borghans J, van Swieten P, Miedema F, *et al.* **Viral replication capacity as a correlate of HLA B57/B5801-associated nonprogressive HIV-1 infection.** *J Immunol* 2007; **179**:3133–3143.
49. Brockman MA, Schneidewind A, Lahaie M, Schmidt A, Miura T, Desouza I, *et al.* **Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A.** *J Virol* 2007; **81**:12608–12618.

PLOS | ONE

RESEARCH ARTICLE

What Triggers a Diagnosis of HIV Infection in the Tokyo Metropolitan Area? Implications for Preventing the Spread of HIV Infection in Japan

Takeshi Nishijima[1]*, Misao Takano[1], Shoko Matsumoto[1,2], Miki Koyama[1], Yuko Sugino[1], Miwa Ogane[1], Kazuko Ikeda[1], Yoshimi Kikuchi[1], Shinichi Oka[1,3], Hiroyuki Gatanaga[1,3]

1 AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan, **2** Graduate School of Public Health, Teikyo University, Tokyo, Japan, **3** Center for AIDS Research, Kumamoto University, Kumamoto, Japan

* tnishiji@acc.ncgm.go.jp

Citation: Nishijima T, Takano M, Matsumoto S, Koyama M, Sugino Y, Ogane M, et al. (2015) What Triggers a Diagnosis of HIV Infection in the Tokyo Metropolitan Area? Implications for Preventing the Spread of HIV Infection in Japan. PLoS ONE 10(11): e0143874. doi:10.1371/journal.pone.0143874

Editor: Philip Anglewicz, Tulane University School of Public Health, UNITED STATES

Received: August 24, 2015

Accepted: November 10, 2015

Published: November 25, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by a Grant-in Aid for AIDS research from the Japanese Ministry of Health, Labour, and Welfare (H23-AIDS-001). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Background

Japan has not succeeded in reducing the annual number of new HIV-infected patients, although the prevalence of HIV infection is low (0.02%).

Methods

A single-center observational study was conducted at the largest HIV clinic in Tokyo, which treats 15% of the total patients in Japan, to determine the reasons for having diagnostic tests in newly infected individuals. HIV-infected patients who visited our clinic for the first time between 2011 and 2014 were analyzed.

Results

The 598 study patients comprised one-third of the total reported number of new patients in Tokyo during the study period. 76% were Japanese MSM. The reasons for being tested which led to the diagnosis was voluntary testing in 32%, existing diseases in 53% (AIDS-defining diseases in 22%, sexually transmitted infections (STI) in 8%, diseases other than AIDS or STIs in 23%) and routine pre-surgery or on admission screening in 15%. 52% and 74% of the study patients and patients presented with AIDS, respectively, had never been tested. The median CD4 count in patients with history of previous testing (315/μL) was significantly higher than that of patients who had never been tested (203/μL, $p<0.001$).

Conclusions

Only 32% of the newly HIV diagnosed patients were diagnosed because of voluntary testing, and 53% were diagnosed due to presence of other diseases. These results remain unchanged from our previous report 10 years earlier (2000–2004) on newly diagnosed

patients at the same clinic. HIV testing has not been widely used by newly diagnosed patients in the Tokyo metropolitan area.

Introduction

The advent and evolution of the combination antiretroviral therapy (cART) has substantially improved the prognosis of patients with HIV infection [1]. Furthermore, suppression of HIV viremia with cART does not only improve the prognosis of HIV-infected individuals regardless of their CD4 count [2,3], but also prevent the sexual transmission of HIV regardless of heterosexual or homosexual contact [4,5]. This "treatment as prevention" strategy is regarded as the main force in the attempt to prevent HIV transmission worldwide [6–8], since a preventive vaccine is currently unavailable. Based on this strategy, American Health and Human Services Guidelines recommend cART for all HIV-infected individuals and even the WHO guidelines were updated in 2015 to recommend that "ART should be initiated in all adults living with HIV at any CD4 cell count", with the hope of reducing the number of newly infected individuals [8,9]. At this stage, the importance of promoting HIV testing for individual at risk for HIV infection cannot be overemphasized, because diagnosis in the early stage of HIV infection and prompt introduction of cART will improve the prognosis of infected individuals [10,11] and at the same time prevent the transmission of HIV [4]. Together with other means, such as circumcision, condom usage, and needle and syringe program, efforts towards the prevention of HIV epidemic appear to be fruitful, with a reported decrease in the number of newly infected individual worldwide from 3.4 million in 2001 to 2.3 million in 2012 [12].

In Japan, however, efforts towards prevention of HIV epidemic have not been successful. Physicians are required to report a diagnosed HIV-infected patient by law, and since the first case was reported in the 1980s, the number of newly infected individuals continues to rise, and was 1,500 per year in 2007. Since 2007 approximately 1,500 new infections are being diagnosed every year for the last 8 years [13]. In Japan, majority of HIV-infected individuals are Japanese men who have sex with men (MSM) and only a few are injection drug users and females, and the majority reside in the metropolitan areas of Tokyo, Osaka, and Nagoya [13]. Especially the Tokyo metropolitan area, including Tokyo, Kanagawa, Saitama, and Chiba prefectures have the largest number of HIV infected individuals, as 46% of the total reported number of HIV-infected individuals in Japan have been reported in this area [13]. It is also noteworthy that approximately 30% of the patients were diagnosed in the advanced stage of HIV infection, following the development of AIDS-defining diseases [13].

Based on the abovementioned background, the present study was designed to understand the reasons for having diagnostic tests in newly infected individuals visiting the largest HIV clinic in Japan located in the Tokyo metropolitan area. Such understanding could help design effective intervention policies to prevent the spread of HIV infection in Japan.

Methods

Study design, setting, and participants

We conducted a single-center observational study to elucidate the reasons for having HIV diagnostic testing in newly infected individuals in the Tokyo metropolitan area. Our clinic, AIDS Clinical Center, National Center for Global Health and Medicine (NCGM), Tokyo, is the largest referral center for HIV infection in Japan [14] with approximately 4,000 registered patients. In this regard, the total reported number of patients with HIV infection in Japan at the end of

2014 was 26,000 [13]. Of these, 6,408 resided in Tokyo, and 12,032 in the Tokyo metropolitan area, including Tokyo, Kanagawa, Saitama, and Chiba prefectures. Thus, our clinic managed approximately 15% of the total HIV infected patients in Japan, and substantially higher percentage of HIV-infected patients in the Tokyo metropolitan area. The following inclusion criteria were applied for enrollment of patients in the study: 1) HIV-infected patients with over 19 years of age who visited our clinic for the first time between January 2011 and December 2014, 2) diagnosis of HIV infection was established preceding and within one year from the first visit to the clinic, thus including only recently diagnosed patients. All HIV-1-infected patients who visited our clinic for the first time were tested with the combined HIV-1 antigen and HIV-1/2 antibody fourth-generation assay, HIV-1 RNA PCR assay, and HIV-1 Western Blot, and only those who were confirmed to be HIV-1 positive based on the results of these assays were included as the study patients. Following exclusion criteria were applied: 1) patients who were vertically infected with HIV-1, 2) patients whose reason for undergoing the diagnostic tests for HIV infection was unknown, and 3) patients who did not undergo routine blood and urine tests in the first visit, such as those who visited the clinic for a second opinion.

The study protocol was approved by the Human Research Ethics Committee of National Center for Global Health and Medicine. Informed consent was waived because this study solely used the data gained from clinical practice. The clinical records were de-identified and analyzed anonymously. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Definitions and measurements

The reasons for HIV diagnostic testing, the day (if not available, the month) of diagnosis of HIV infection, history of AIDS-defining diseases, perceived route of transmission, sexual orientation (men were asked whether they have sex with men), history of previous HIV testing, treatment status of HIV infection (treatment-naïve or experienced), and history of HBV vaccination, as well as the basic characteristics, such as age, sex, and ethnicity, were collected through a structured interview conducted at the first visit as part of routine clinical practice by the nurses specializing at the HIV outpatient care [15], and also through a structured interview by the treating physician. The patients who voluntarily tested for HIV infection were also asked whether they had tested because their sexual partners were diagnosed of HIV infection. Blood samples were also routinely collected at the first visit for CD4 count, HIV-1 RNA viral load, HIV-1 western blot testing, hepatitis B surface antigen (HBsAg), antibody to HBsAg (anti-HBs), antibody to hepatitis B core antigen (anti-HBc), hepatitis C antibody (anti-HCV), serum quantitative *Treponema pallidum* hemagglutination (TPHA) and rapid plasma reagin (RPR) test, and anti-Entamoeba histolytica antibody (anti-Eh).

The reasons for having HIV diagnostic tests were classified into 5 categories: (1) patients who were voluntarily tested (the voluntary group); voluntary testing was applied to those who visited public health center or other healthcare facilities for the purpose of receiving HIV diagnostic tests, or those who performed home-based self-test. This group also included subjects who were voluntary tested and later were diagnosed with AIDS-defining diseases. (2) occurrence of AIDS-defining diseases (23 diseases set by the Japanese Ministry of Health, Labour, and Welfare [16]) (the AIDS group), (3) diagnosis with sexually transmitted infections (STI) (the STI group), (4) occurrence of diseases other than AIDS-defining diseases and STIs (the non-AIDS disease group), (5) patients diagnosed incidentally after routine screening, such as before surgery, on admission to the hospital, or antenatal screening (the screening group). Acute HIV infection was defined as positive HIV nucleic acid testing and a non-reactive or indeterminate western blot [17]. Active syphilis infection which required treatment was

defined as patients with both serum RPR titer ≥8 and positive TPHA result [18]. History of syphilis was defined as patients with positive TPHA. Chronic HBV infection was defined as patients with positive HBsAg, whereas exposure to HBV was defined as those with either positive HBsAg, anti-HBsAg, or anti-HBc [19], because in Japan, universal HBV vaccination has not been introduced, except for health care professionals [20].

Statistical analysis

Baseline characteristics were described for the entire study patients. The median CD4 count of patients with history of previous HIV testing and no previous testing were compared with the Mann-Whitney U test. Statistical significance was defined as two-sided p values <0.05. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 21.0 (SPSS, Chicago, IL).

Results

A total of 822 patients with HIV infection visited our clinic for the first time during the study period (Fig 1). Of them, 598 patients were analyzed as the study patients. The study patients comprised one-third of the total reported number of newly diagnosed HIV-infected individuals in Tokyo during the study period [13]. 95% of the study patients were males, and 88% were Japanese (Table 1). The median age was 37 [interquartile range (IQR) 30–44)], and 24% were in their 20's while 59% were <40 years old. Furthermore, 84% were MSM, and Japanese MSM comprised 76% of the study subjects. 96% were treatment-naïve, with a median CD4 count of 231 /μL (86–390), and HIV viral load of 4.92 $\log_{10}$copies/mL (4.38–5.44). 44 (7%) patients presented with acute HIV infection.

The reasons for undergoing the diagnostic tests for HIV infection was voluntary testing in 190 (31.8%), AIDS-defining diseases in 129 (21.6%), STIs in 50 (8.4%), diseases other than AIDS or STIs in 139 (23.2%), and before surgery, on admission, or antenatal routine screening in 90 (15%). Of the voluntary group, 47 (25%) individuals requested the tests because their partners had been diagnosed with HIV infection, and only 6 (3.1%) patients used a home-based self-test kit, including a mailing kit. The percentage of patients who were diagnosed of HIV infection because of voluntary testing among MSM was significantly higher than that among non-MSM [170 (34%) of 502 versus 20 (21%) of 96, p = 0.012)]. 52% of the study patients have never been tested previously for HIV infection, and among those with AIDS-defining diseases, 74% have never been tested previously. Furthermore, among patients who have never been tested previously for HIV infection, 71 (27%) had a history of STIs, including syphilis, hepatitis A, B, or C, gonorrhea, genital herpes, chlamydia, condyloma acuminatum, amoebiasis, and pubic lice. However, they were not screened for HIV infection at the time of STI presentation.

The median CD4 count at the first visit was 338 /μL (IQR 211–467) in the voluntary testing group, 292 /μL (IQR 147–408) in the screening group, 259 /μL (IQR 155–415) in the STI group, 234 /μL (IQR 122–392) in the non-AIDS disease group, and was the lowest in the AIDS group [54 /μL (IQR 23–98)]. Furthermore, the median CD4 count was significantly higher in patients with history of previous testing (315 /μL, IQR 175–450) than those without (203 /μL, IQR 81–354) (p<0.001, the Mann-Whitney U test).

Active syphilis infection that required treatment was diagnosed in 17% of the study patients, whereas history of syphilis defined by positive TPHA was observed in 34%. HBsAg was positive in 7% of the patients, and 51% were exposed to HBV. HCVAb was positive in 4% of the patients, and anti-Eh antibody was positive in 19%.

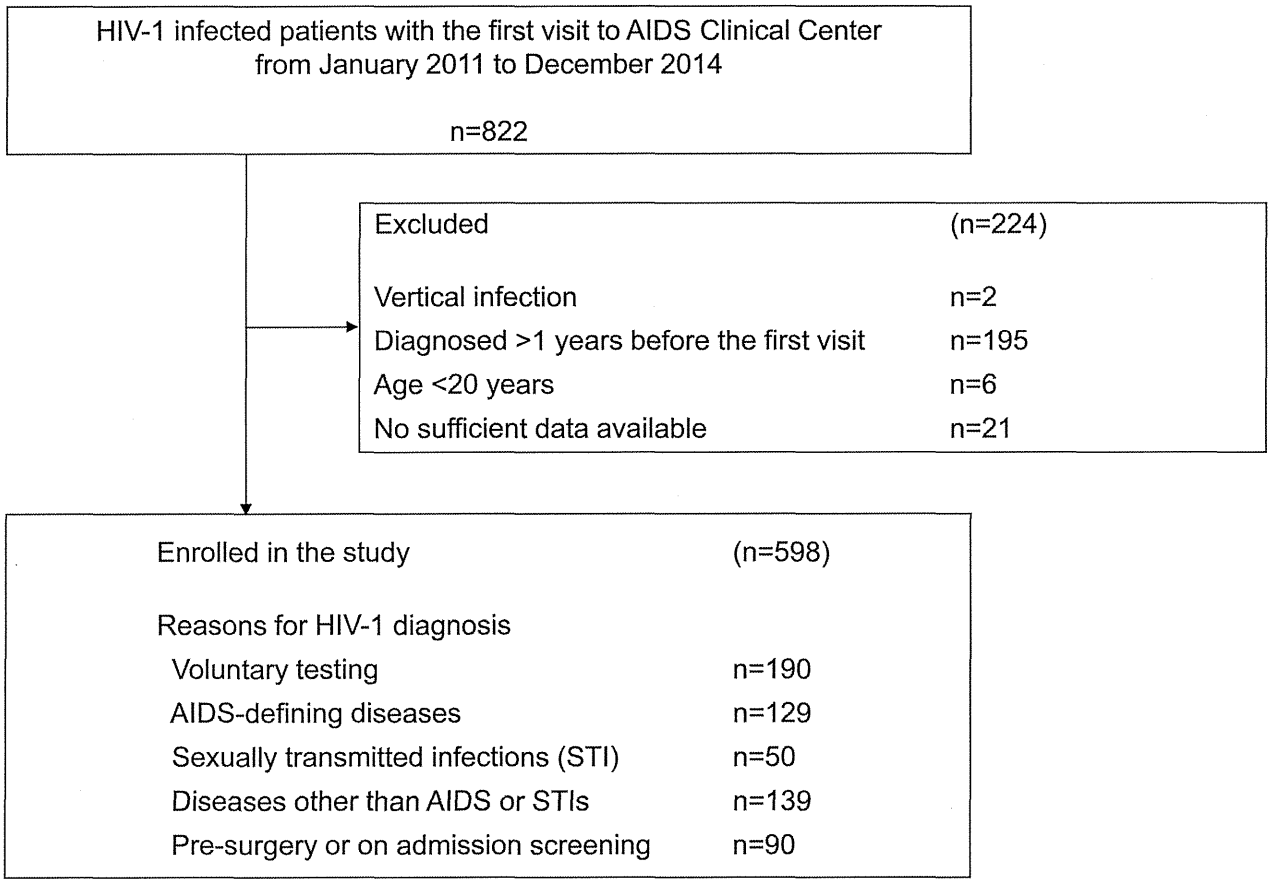

Fig 1. Patient enrollment process.

doi:10.1371/journal.pone.0143874.g001

Discussion

In this largest HIV clinic in Japan where approximately 15% of the total patients in Japan are treated, only 32% of the newly diagnosed patients between 2011 and 2014 were diagnosed with HIV infection because of voluntary testing. Alarmingly, 53% of the newly diagnosed patients underwent testing after the development of other diseases; which were either AIDS-defining diseases (22%), STIs (8%), or diseases other than AIDS and STIs (23%). Furthermore, 15% of the new diagnosis were incidentally made by the routine screening on admission to hospital, before surgery, or antenatal screening. More importantly, 52% of the newly diagnosed patients had never been tested for HIV infection, and this proportion was even higher (74%) among those who presented with AIDS-defining diseases. These results showed that HIV testing has not been widely utilized in newly diagnosed patients with HIV infection, who had been at high risk for HIV acquisition, in the largest HIV clinic in Japan located in the Tokyo metropolitan area.

Early establishment of the diagnosis of HIV infection and early initiation of treatment are both crucial for improvement of prognosis and lessening the spread of infection [10,11,21]. Our study also showed that the median CD4 count of the patients who were voluntarily tested and diagnosed [338 /μL (IQR 211–467)] was higher than that of patients with AIDS [54 /μL (IQR 23–98)] and non-AIDS diseases [234 /μL (IQR 122–392)]. In this regard, various efforts have been made to promote HIV testing in Japan, such as setting up free and anonymous

Table 1. Characteristics of the study patients (n = 598).

	n or median	% or interquartile range
Male sex, n (%)	565	94.5
Age (years)†		
20–29	144	24.1
30–39	210	35.1
40–49	175	29.3
>49	69	11.5
Ethnicity		
Japanese	523	87.5
Asians other than Japanese	41	6.9
Others	34	5.6
CD4 count (/μl)†¶	231	86–390
HIV RNA load (log_{10}/ml)†	4.92	4.38–5.44
Treatment-naive, n (%)	572	95.7
Men who have sex with men	502	83.9
No history of previous HIV testing¶	261	52
AIDS-defining illnesses	165	27.6
Acute HIV infection	44	7.4
Positive anti-Eh antibody¶	109	19
Rapid plasma reagin titer ≥8	103	17.2
Positive TPHA	204	34.1
Positive HCV antibody	22	3.7
Positive HBs antigen	43	7.2
HBV exposure*	306	51.3
Route of transmission		
Homosexual contact	491	82.1
Heterosexual contact	89	14.9
Injection drug or homosexual contact	11	1.8
Unknown	7	1.2

†Median (interquartile range). anti-Eh antibody, anti-entamoeba histolytica antibody; TPHA, *Treponema pallidum* hemagglutination; HCV, hepatitis C virus; HBs antigen, hepatitis B surface antigen

¶CD4 count is missing for one patient, history of previous HIV testing is missing for 96 patients, and anti-Eh antibody is missing for 25 patients.

*Two patients with history of HBV vaccination were excluded.

doi:10.1371/journal.pone.0143874.t001

testing sites at public health centers and other facilities across Japan, creating a website that provides information on HIV testing, publishing HIV testing guidelines for use of healthcare facilities, ensuring availability of rapid HIV tests at private clinics in the high-prevalence area, and outreaching events for sexual minorities to promote diagnostic testing. These efforts were spearheaded mainly by different study groups established by the Japanese Ministry of Health, Labour, and Welfare, in collaboration with various non-governmental organizations [22]. However, the annual reported number of newly infected cases reached its peak in 2007 with 1,500, and has stabilized with approximately 1,500 new patients every year in the last 8 years [13]. It is disappointing that the results of the present study are very similar to our previous report, which investigated the reasons for diagnostic testing in newly infected patients who visited our clinic for the first time between 2000 and 2005 [23]. In that study, the voluntary testing group comprised 35% of newly infected patients, while patients with diseases including AIDS

comprised 52%, and those who were incidentally diagnosed due to routine clinical testing formed 13% (Table 2). One major difference between the present and previous analyses is the percentage of patients without history of previous testing (decreased from 74% to 52%). These results highlight the difficulty in promoting HIV testing among high-risk population, such as MSM, in Japan, which has very low prevalence of HIV infection (prevalence: 0.02%, based on 26,000 reported patients by the end of 2014 [13] among a population of 127 million according to the census conducted in January 2015 [24]).

How can we reduce the number of newly HIV-infected individuals in Japan? Efforts to promote HIV diagnostic testing in high-risk population need to be continued and strengthened, however, it is probably not enough to reduce the number of new infections, as described above. Considering the difficulty of testing a high-risk population in a country with very low prevalence of HIV infection, the "treatment as prevention" strategy, which in principle encourages all HIV-infected individuals to start treatment, might be an efficient way to prevent transmission of HIV. Currently, the Japanese Guidelines for the treatment of HIV infection 2015 recommend initiation of cART in a treatment-naïve patient with CD4 count ≤350 /μL (strong recommendation), with CD4 351–500 /μL (strong/moderate recommendation), and also for those with CD4 count >500 /μL (moderate recommendation based on expert opinion) [25]. However, in Japan, most HIV-infected individuals obtain a certificate that allows them to receive financial assistance for out-of-pocket medical expenditure for cART, and for patients with CD4 count >500 /μL it is not always possible to obtain such assistance [26], and many such patients hesitate to start cART until CD4 count decreases to <500 /μL because of financial concern. It is desirable to remove this CD4 threshold from the requirement for obtaining financial assistance if "treatment as prevention" strategy is to be further promoted for the prevention of HIV epidemic in Japan, and to improve the prognosis of patients with CD4 count >500 /μL by early initiation of cART [2,3]. This "treatment as prevention" strategy is further backed up by a recently published article by Nosyk and colleagues which showed that treatment-for-all strategy in British Columbia, Canada, has been successful in not only reducing the number of new HIV-1 infected patients, but also being cost effective and furthermore, will be cost saving in the long-term [27].

Another important strategy for the diagnosis of HIV-infected patients in the early stage of infection is partner notification, counseling, and testing of sex partners of patients newly diagnosed with HIV infection, because such partners are at very high risk for HIV infection and diagnostic testing will benefit health of such partners [28]. Provider-assisted partner counseling and testing at our clinic has been very successful, as we had reported that 17 out of 86 (20%) of tested partners of patients with newly diagnosed infection were found to have HIV infection [29]. The present study also showed that among the patients who were voluntary tested for HIV, 25% had such test because of partner notification, suggesting the importance of such strategy.

Table 2. Comparison of reasons for HIV diagnostic testing in newly diagnosed patients between 2000–2005 and 2011–2014 time periods.

Reasons for HIV diagnostic testing	2000–2005 (n = 654)		2011–2014 (n = 598)	
	n	%	N	%
Voluntary testing	230	35	190	32
Presence of diseases (AIDS, non AIDS, or STIs)	338	52	318	53
Routine before-surgery or on admission screening	86	13	90	15

The data on newly diagnosed patients between 2000 and 2005 were cited from our previous study [23].

doi:10.1371/journal.pone.0143874.t002

Moreover, patients who present with STIs, especially syphilis and horizontally infected HBV, should be exhaustively tested for HIV infection, since such STIs are highly prevalent among patients with newly diagnosed HIV infection, as shown in the present study. The result that among the patients who had never been tested previously for HIV infection, 27% of such patients actually had a history of STIs but they were not tested for HIV, warrants the need for further raising awareness in healthcare personnel including primary care physicians.

In this study, only 3.2% of the patient who voluntarily tested for HIV infection used home-based HIV self-test. Consistent with this result, in Japan, home-based HIV self-test has a limited role in diagnosis of HIV infection, where the mainstream of the home-based test is "a mailing HIV self-check kit". The reported number of usage of such kit is increasing from 39,868 in 2006 to 73,863 in 2013, however, the reported number of positive result has been stable from 221 in 2006 to 192 in 2013 [30]. This mailing kit is a screening assay and cannot make definitive diagnosis of HIV infection. Also, they are expected to yield many false positive results due to low prevalence of HIV infection in Japan.

The strength of the present study include the uniqueness of detailed data on the reasons for diagnostic testing in newly infected individuals in Japan, a large-scale study which included one-third of the total reported number of newly diagnosed patients in Tokyo, and comparability of the present study with those of a previous study conducted by the same institution about 10 years earlier. Apart from the strengths of this study, few limitations need to be acknowledged. Being a single-center study, selection bias is not avoidable. However, as described above, our clinic treats approximately 15% of the patients in Japan, and the study patients covered one-third of the total newly diagnosed patients in Tokyo, where prevalence of HIV infection is highest [13]. Furthermore, the study population truly represents HIV-infected patients in Japan as a whole. For example, among the 1,546 newly reported HIV-infected individuals in 2014, 88.5% were Japanese males, 29.4% of the patients presented with AIDS-defining diseases, and 67.7% were infected through homosexual contact [13].

In conclusion, in the largest HIV clinic in Japan, only 32% of the newly diagnosed HIV-infected patients between 2011 and 2014 were diagnosed based on voluntary testing, and 53% were diagnosed because they had AIDS, non-AIDS diseases, or STIs. Furthermore, 52% of the newly diagnosed patients have never been tested for HIV infection. Importantly, these results largely remain unchanged from similar data analyzed 10 years ago by the same clinic [23]. While promoting diagnostic testing for the at-risk population for HIV infection remains important, the practice of "treatment as prevention" strategy needs to be encouraged in order to reduce the spread of HIV infection in Japan.

Acknowledgments

The authors thank Katsuji Teruya, Kunihisa Tsukada, Junko Tanuma, Haruhito Honda, Ei Kinai, Koji Watanabe, Takahiro Aoki, Daisuke Mizushima, Taiichiro Kobayashi, Yasuaki Yanagawa, Satoshi Shibata, Haruka Uemura, Yuki Yamada, Jongmi Seo, Beni Taniguchi, Hitomi Suzuki, Mari Kinoshita, and Naomi Nakaie. The authors also thank Akiko Nakano, the study coordinator, and all the other staff at the AIDS Clinical Center for their help in the completion of this study.

Author Contributions

Conceived and designed the experiments: TN MT SO HG. Performed the experiments: MK YS MO KI. Analyzed the data: TN MT. Contributed reagents/materials/analysis tools: YK. Wrote the paper: TN MT SM SO HG.

References

1. Helleberg M, May MT, Ingle SM, Dabis F, Reiss P, Fatkenheuer G, et al. (2015) Smoking and life expectancy among HIV-infected individuals on antiretroviral therapy in Europe and North America. AIDS 29: 221–229. doi: 10.1097/QAD.0000000000000540 PMID: 25426809
2. INSIGHT START Study Group (2015) Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection. N Engl J Med 373: 795–807. doi: 10.1056/NEJMoa1506816 PMID: 26192873
3. TEMPRANO ANRS 12136 Study Group (2015) A Trial of Early Antiretrovirals and Isoniazid Preventive Therapy in Africa. N Engl J Med 373: 808–22. doi: 10.1056/NEJMoa1507198 PMID: 26193126
4. Cohen MS, Chen YQ, McCauley M, Gamble T, Hosseinipour MC, Kumarasamy N, et al. (2011) Prevention of HIV-1 infection with early antiretroviral therapy. N Engl J Med 365: 493–505. doi: 10.1056/NEJMoa1105243 PMID: 21767103
5. Rodger A, Bruun T, Cambiano V, Bruun T, Vernazza P, Collins S, Estrada V, et al. HIV Transmission Risk Through Condomless Sex If HIV+ Partner On Suppressive ART: PARTNER Study. Conference on Retroviruses and Opportunistic Infections 2014 Abstract 153LB.
6. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. Available: http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf. Accessed June 30 2015.
7. World Health Organization. Consolidated Guidelines on the use of antiretroviral drugs for treating and preventing HIV infection. Recommendations for a public health approach June 2013.
8. World Health Organization Guideline on when to start antiretroviral therapy and on pre-exposure prophylaxis for HIV September 2015: Available: http://www.who.int/hiv/pub/guidelines/earlyrelease-arv/en/
9. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. Available: http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf.
10. Laanani M, Ghosn J, Essat A, Melard A, Seng R, Gousset M, et al. (2015) Impact of the Timing of Initiation of Antiretroviral Therapy During Primary HIV-1 Infection on the Decay of Cell-Associated HIV-DNA. Clin Infect Dis 60: 1715–1721. doi: 10.1093/cid/civ171 PMID: 25737374
11. Le T, Wright EJ, Smith DM, He W, Catano G, Okulicz JF, et al. (2013) Enhanced CD4+ T-cell recovery with earlier HIV-1 antiretroviral therapy. N Engl J Med 368: 218–230. doi: 10.1056/NEJMoa1110187 PMID: 23323898
12. UNAIDS 2013 Global Fact Sheet. Available: http://www.unaids.org/en/resources/campaigns/globalreport2013/factsheet.pdf.
13. AIDS Surveillance Committee. The Japanese Ministry of Health, Labour, and Welfare. Available: http://api-net.jfap.or.jp/status/2013/13nenpo/nenpo_menu.htm. in Japanese.
14. Nishijima T, Komatsu H, Gatanaga H, Aoki T, Watanabe K, Kinai E, et al. (2011) Impact of small body weight on tenofovir-associated renal dysfunction in HIV-infected patients: a retrospective cohort study of Japanese patients. PLoS One 6: e22661. doi: 10.1371/journal.pone.0022661 PMID: 21799928
15. Nishijima T, Gatanaga H, Komatsu H, Takano M, Ogane M, Ikeda K, et al. (2013) Illicit drug use is a significant risk factor for loss to follow up in patients with HIV-1 infection at a large urban HIV clinic in Tokyo. PLoS One 8: e72310. doi: 10.1371/journal.pone.0072310 PMID: 23951307
16. The Japanese Ministry of Health, Labour and Welfare. Available: http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansenshou11/01-05-07.html in Japanese.
17. Ananworanich J, Fletcher JL, Pinyakorn S, van Griensven F, Vandergeeten C, Schuetz A, et al. (2013) A novel acute HIV infection staging system based on 4th generation immunoassay. Retrovirology 10: 56. doi: 10.1186/1742-4690-10-56 PMID: 23718762
18. Tanizaki R, Nishijima T, Aoki T, Teruya K, Kikuchi Y, Oka S, et al. (2015) High-dose Oral Amoxicillin Plus Probenecid Is Highly Effective for Syphilis in Patients with HIV Infection. Clin Infect Dis.
19. Gatanaga H, Hayashida T, Tanuma J, Oka S (2013) Prophylactic effect of antiretroviral therapy on hepatitis B virus infection. Clin Infect Dis 56: 1812–1819. doi: 10.1093/cid/cit145 PMID: 23487374
20. Komatsu H (2014) Hepatitis B virus: where do we stand and what is the next step for eradication? World J Gastroenterol 20: 8998–9016. doi: 10.3748/wjg.v20.i27.8998 PMID: 25083074
21. Ransome Y, Terzian A, Addison D, Braunstein S, Myers J, Abraham B, et al. (2015) Expanded HIV testing coverage is associated with decreases in late HIV diagnoses. AIDS 29: 1369–1378. doi: 10.1097/QAD.0000000000000684 PMID: 26091296
22. Research Committee on the research of constructing the system for HIV testing, the Japanese Ministry of Health, Labour, and Welfare. Available: https://mhlw-grants.niph.go.jp/niph/search/NIDD00.do?resrchNum=200300565A. in Japanese.

23. Takano M, Okada M, Oka S, Wagatsuma Y (2012) The relationship between HIV testing and CD4 counts at HIV diagnosis among newly diagnosed HIV-1 patients in Japan. Int J STD AIDS 23: 262–266. doi: 10.1258/ijsa.2009.009493 PMID: 22581950

24. Statistics Bureau, the Japanese Ministry of Internal Affairs and Communications. Estimated population of Japan as of 1st of Janurary, 2015. Available: http://www.stat.go.jp/data/jinsui/new.htm. in Japanese.

25. The Guidelines for the Treatment of HIV Infection, March 2015 version. The Japanese Ministry of Health, Labour and Welfare. 1–159. Available: http://www.haart-support.jp/guideline.htm. Accessed 20 Decenber 2013.

26. Reports on the certification of disabilities due to immune deficiency by HIV. the Japanese Ministry of Health, Labour, and Welfare. 1997. Available: http://www1.mhlw.go.jp/shingi/s1216-3.html. in Japanese

27. Nosyk B, Min JE, Lima VD, Hogg RS, Montaner JS (2015) Cost-effectiveness of population-level expansion of highly active antiretroviral treatment for HIV in British Columbia, Canada: a modelling study. Lancet HIV 2: e393–400. doi: 10.1016/S2352-3018(15)00127-7 PMID: 26423553

28. Brown LB, Miller WC, Kamanga G, Kaufman JS, Pettifor A, Dominik RC, et al. (2012) Predicting partner HIV testing and counseling following a partner notification intervention. AIDS Behav 16: 1148–1155. doi: 10.1007/s10461-011-0094-9 PMID: 22120879

29. Seo J, Takeda K, Yamada Y, Shimada M, Ikeda K, Kukichi Y, et al. The HIV testing for sexual partners of newly diagnosed HIV-infected patients. Part two. 24th Annual Meeting of the Japanese Society for AIDS Research. 2010 Abstract WS3-014.

30. Research Committee on the research of constructing the system for HIV testing, the Japanese Ministry of Health, Labour, and Welfare. Available: http://www.mhlw.go.jp/file/05-Shingikai-10901000-Kenkoukyoku-Soumuka/0000042611.pdf.

Medicine®

OBSERVATIONAL STUDY

OPEN

Upper Gastrointestinal Symptoms Predictive of Candida Esophagitis and Erosive Esophagitis in HIV and Non-HIV Patients

An Endoscopy-Based Cross-Sectional Study of 6011 Patients

Yuta Takahashi, MD, Naoyoshi Nagata, MD, Takuro Shimbo, PhD, Takeshi Nishijima, PhD, Koji Watanabe, MD, Tomonori Aoki, MD, Katsunori Sekine, MD, Hidetaka Okubo, MD, Kazuhiro Watanabe, MD, Toshiyuki Sakurai, MD, Chizu Yokoi, PhD, Akio Mimori, PhD, Shinichi Oka, PhD, Naomi Uemura, PhD, and Junichi Akiyama, MD

Abstract: Upper gastrointestinal (GI) symptoms are common in both HIV and non-HIV-infected patients, but the difference of GI symptom severity between 2 groups remains unknown. Candida esophagitis and erosive esophagitis, 2 major types of esophagitis, are seen in both HIV and non-HIV-infected patients, but differences in GI symptoms that are predictive of esophagitis between 2 groups remain unknown. We aimed to determine whether GI symptoms differ between HIV-infected and non-HIV-infected patients, and identify specific symptoms of candida esophagitis and erosive esophagitis between 2 groups.

We prospectively enrolled 6011 patients (HIV, 430; non-HIV, 5581) who underwent endoscopy and completed questionnaires. Nine upper GI symptoms (epigastric pain, heartburn, acid regurgitation, hunger cramps, nausea, early satiety, belching, dysphagia, and odynophagia) were evaluated using a 7-point Likert scale. Associations between esophagitis and symptoms were analyzed by the multivariate logistic regression model adjusted for age, sex, and proton pump inhibitors.

Endoscopy revealed GI-organic diseases in 33.4% (2010/6.011) of patients. The prevalence of candida esophagitis and erosive esophagitis was 11.2% and 12.1% in HIV-infected patients, respectively, whereas it was 2.9% and 10.7% in non-HIV-infected patients, respectively. After excluding GI-organic diseases, HIV-infected patients had significantly ($P < 0.05$) higher symptom scores for heartburn, hunger cramps, nausea, early satiety, belching, dysphagia, and odynophagia than non-HIV-infected patients. In HIV-infected patients, any symptom was not significantly associated with CD4 cell count. In multivariate analysis, none of the 9 GI symptoms were associated with candida esophagitis in HIV-infected patients, whereas dysphagia and odynophagia were independently ($P < 0.05$) associated with candida esophagitis in non-HIV-infected patients. However, heartburn and acid regurgitation were independently ($P < 0.05$) associated with erosive esophagitis in both patient groups. The internal consistency test using Cronbach's α revealed that the 9 symptom scores were reliable in both HIV (α, 0.86) and non-HIV-infected patients (α, 0.85).

This large-scale endoscopy-based study showed that HIV-infected patients have greater GI symptom scores compared with non-HIV-infected patients even after excluding GI-organic diseases. None of the upper GI symptoms predict candida esophagitis in HIV-infected patients, but dysphagia and odynophagia predict candida esophagitis in non-HIV-infected patients. Heartburn and acid regurgitation predict erosive esophagitis in both patient groups.

(*Medicine* 94(47):e2138)

Abbreviations: ADL = activities of daily living, CE = *Candida* esophagitis, CI = confidential intervals, EE = erosive esophagitis, GI = gastrointestinal, GSRS = GI symptom rating scale, HAART = highly active antiretroviral therapy, IQR = interquartile range, OR = odds ratio, PPI = proton-pump inhibitor.

INTRODUCTION

Upper gastrointestinal (GI) symptoms are common in both HIV and non-HIV-infected patients.[1–3] In the current era of highly active antiretroviral therapy (HAART), the incidence of many opportunistic or AIDS-defining diseases has been reduced.[4] Thus, the characteristics of upper GI disease in HIV-infected patients are likely to be similar to those in the general population.[5]

Candida esophagitis (CE) and erosive esophagitis (EE), 2 major types of esophagitis, are seen in both HIV and non-HIV-infected patients.[6,7] A variety of symptoms including heartburn, acid regurgitation, hunger cramps, nausea, early satiety, belching, dysphagia, and odynophagia have been reported to predict esophagitis.[1,8–11] However, previous studies were not prospective in design, did not use validated scales, or did not exclude GI-organic diseases despite the presence of typical esophageal symptoms suggestive of these diseases.[1,8–11] Elucidating disease-specific GI symptoms may allow physicians to avoid delays in diagnosis and prevent poor outcomes or overuse of endoscopy, but it remains unclear which symptoms can predict

Editor: Zarko Babic.
Received: July 14, 2015; revised: September 13, 2015; accepted: October 30, 2015.
From the Department of Gastroenterology and Hepatology, National Center for Global Health and Medicine, Tokyo (YT, NN, TA, KS, HO, KW, TS, CY, JA); Ohta Nishinouchi Hospital, Fukushima (TS); Division of AIDS Clinical Center, National Center for Global Health and Medicine (TN, KW, SO); Division of Rheumatic Diseases, National Center for Global Health and Medicine, Tokyo (AM); and Department of Gastroenterology and Hepatology, National Center for Global Health and Medicine, Kohnodai Hospital, Chiba, Japan (NU).
Correspondence: Naoyoshi Nagata, Department of Gastroenterology and Hepatology, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo, Japan (e-mail: nnagata_ncgm@yahoo.co.jp).
Funding: this work was supported by a Grant from the National Center for Global Health and Medicine (21-101) and a Grant-in-Aid for AIDS research from the Ministry of Health, Labour, and Welfare of Japan (H26-003). Funders had no roles in the study design; in the collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.
The authors have no conflicts of interest to disclose.

ISSN: 0025-7974
DOI: 10.1097/MD.0000000000002138

the 2 types of esophagitis among HIV and non-HIV infected patients.

To address this issue, we evaluated 9 specific upper GI symptoms using a 7-point Likert scale on the day of pre-endoscopy, and diagnosed various upper GI diseases by endoscopy in a large number of HIV and non-HIV-infected patients. The aim was to determine whether upper GI symptoms were different between HIV-infected and non-HIV-infected patients, and to investigate symptoms that are predictive of CE and EE in patients with or without HIV infection.

METHODS

Study Design, Setting, and Participants

We conducted a hospital-based, prospective, cross-sectional study at the endoscopy unit of the National Center for Global Health and Medicine (NCGM; Tokyo, Japan) between September 2009 and April 2014. NCGM has 900 beds and is the largest referral center for HIV/AIDS in Japan. Inclusion criteria were as follows: (i) age $\geq$18 years; (ii) Japanese nationality; (iii) continual or severe upper GI symptoms; (iv) screening for GI cancer. In Japan, where there is a high incidence of gastric cancer, endoscopy is frequently performed for gastric cancer screening. Exclusion criteria were as follows: (i) no informed consent obtained; (ii) unknown medication use; (iii) dependent on activities of daily living (ADL); (iv) inability to understand written documents; (v) use of any antifungal drug within 1 month before endoscopy; and (vi) urgent or early endoscopy for acute GI bleeding.

This study was approved by the ethics committee of the National Center for Global Health and Medicine (No. 1440), and written informed consent was obtained from all patients prior to endoscopy.

Data Sources and Measurement

A detailed questionnaire was completed at the endoscopy unit on the day of pre-endoscopy.[12,13] Use of a proton-pump inhibitor (PPI) was defined as intermittent or regular administration within 1 month before the interview. All patients underwent serological testing for HIV before endoscopy. CD4 cell counts in the 1 month before or after endoscopy were obtained from the medical records. Information regarding history of HAART was collected from pre-endoscopy medical records.

Upper GI symptoms were evaluated using the modified GI symptom rating scale (GSRS). The modified GSRS consists of the original GSRS (epigastric pain, heartburn, acid regurgitation, hunger cramps, and nausea) plus early satiety, belching, dysphagia, and odynophagia, and assesses the 9 symptoms using a 7-point Likert scale (1, none at all; 2, minor; 3, mild; 4, moderate; 5, moderately severe; 6, severe; and 7, very severe).[13,14] The reliability and validity of the GSRS in the assessment of functional GI disease are well documented.[15]

Diagnosis of Upper GI Disease and Candida Esophagitis

A high-resolution scope (GIF-H260, Olympus Corp., Tokyo, Japan) was used for the diagnosis of upper GI disease. Well-trained staff who were blinded to the questionnaire results performed the endoscopy. When abnormal findings were detected on endoscopy, biopsy, or endoscopic mucosal resection was performed. All removed specimens were evaluated by expert pathologists (>10 years' experience) for making the final diagnoses of upper GI disease. A diagnosis of CE was made if white esophageal plaques detected on endoscopy could not be washed away [10] and pathological assessment with hematoxylin and eosin and periodic acid-Schiff staining or culture for *Candida* species confirmed the clinical findings.[12,13] The diagnosis of EE was based on the presence of circumferential mucosal breaks in the esophagus.[16] "Organic GI disease" included CE, erosive esophagitis, ulcer, early cancer, advanced cancer, other malignancy, and post GI resection on endoscopy.

Statistical Analysis

Baseline characteristics were compared using the Mann–Whitney *U* test or Pearson's chi-square test (Fisher's exact test) for quantitative or qualitative variables, respectively.

To determine whether HIV infection is a risk for upper GI symptoms, associations between HIV infection and upper GI symptom scores were analyzed by univariate and multivariate rank ordered logistic modeling after excluding GI-organic diseases on endoscopy.

To identify predictive symptoms of CE, we adjusted for age, sex, and PPI, which were factors significantly associated with both CE and EE in the univariate analysis and were reported to affect upper GI symptoms.[17] These associations were evaluated after excluding GI-organic disease on endoscopy. We used univariate and multivariate logistic regression models and estimated the odds ratios (OR) and 95% confidential intervals (CI) of each symptoms. Individuals were classified as having positive upper GI symptoms if they scored $\geq$2 for each item on the GSRS.[18]

To evaluate the reliability of GSRS, we analyzed internal consistency. Cronbach's α was used for measurement of internal consistency of the 9 GSRS items. α Values were interpreted as follows: $\geq$0.90, excellent agreement; $0.9 > \alpha \geq 0.80$, good agreement; $0.8 > \alpha \geq 0.7$, acceptable; $0.7 > \alpha \geq 0.6$, questionable; $0.6 > \alpha \geq 0.5$, poor; and $\alpha < 0.5$, unacceptable. $P < 0.05$ was considered statistically significant. All statistical analysis was performed using Stata version 13 software (StataCorp, College Station, TX).

RESULTS

Participants

During the study period, 9337 participants who underwent upper endoscopy were met the inclusion criteria. Of these, 3331 participants were excluded in accordance with the exclusion criteria, the remaining 6011 patients who underwent endoscopy and completed the questionnaire were enrolled in the study.

Baseline Characteristics of HIV and non-HIV-infected Patients

Differences in baseline characteristics between HIV-infected and non-HIV-infected patients are shown in Table 1. The prevalence of CE and EE was 11.2% (48/430) and 12.1% (52/430) in HIV-infected patients, respectively, whereas it was 2.9% (163/5581) and 10.7% (599/5581) in non-HIV-infected patients, respectively. Endoscopy revealed upper GI-organic diseases in 33.4% (2010/6.011) of patients. No significant difference was observed in endoscopy indication, any organic GI disease, EE, esophageal cancer or malignancy, gastric ulcer, advanced gastric cancer, and duodenal ulcer between groups (Table 1). Factors positively associated with HIV infection were younger age, male sex, CE, esophageal ulcer, and gastric malignancy, whereas the factors negatively associated with HIV infection were PPI use, early esophageal cancer, early

TABLE 1. Patient Characteristics in HIV-Infected and Non-HIV-Infected Patients (n = 6011)

	HIV (n = 430)	Non-HIV (n = 5581)	*P* Value
Age (years)	46.7 ± 11.7	60.6 ± 13.9	<0.001
Sex, male	409 (95.1)	2988 (53.5)	<0.001
Use of proton pump inhibitor	54 (12.6)	1624 (29.1)	<0.001
CD4 cell count/μL, median (IQR)	405 (222, 584)		
CD4 cell count <200/μL	100 (23.3)		
History of HAART	342 (79.5)		
Duration of HAART (months)	79.0 ± 60.0		
Indication of endoscopy, cancer screening	395 (91.9)	5155 (92.4)	0.704
Organic GI disease	140 (32.6)	1870 (33.5)	0.688
Candida esophagitis	48 (11.2)	163 (2.9)	<0.001
Erosive esophagitis	52 (12.1)	599 (10.7)	0.382
Esophageal ulcer	12 (2.8)	21 (0.4)	<0.001
Early esophageal cancer	1 (0.2)	130 (2.3)	0.004
Advanced esophageal cancer	0 (0)	33 (0.6)	0.110
Esophageal malignancy	3 (0.7)	18 (0.3)	0.204
Postesophageal resection	0 (0)	27 (0.5)	0.148
Gastric ulcer	14 (3.3)	257 (4.6)	0.194
Early gastric cancer	11 (2.6)	305 (5.5)	0.009
Advanced gastric cancer	0 (0)	32 (0.57)	0.115
Gastric malignancy	20 (4.7)	154 (2.8)	0.024
Postgastric resection	4 (0.9)	266 (4.8)	<0.001
Duodenal ulcer	9 (2.1)	91 (1.6)	0.470
Duodenal malignancy	0 (0)	78 (1.4)	0.014

Data are presented as number (%). Values presented with plus/minus sign are means ± SD. Values in parentheses represent 95% confidential intervals. *P* values are derived from a comparison between patients with HIV and those without HIV.

GI = gastrointestinal, HAART = highly active antiretroviral therapy, HIV = human immunodeficiency virus, IQR = interquartile range, SD = standard deviation.

gastric cancer, post-gastric resection, and duodenal malignancy (Table 1).

Differences in Upper GI Symptoms Between HIV-Infected and non-HIV-infected Patients

Symptom scores for heartburn, hunger cramps, nausea, early satiety, or odynophagia in HIV-infected patients were significantly higher than those in non-HIV-infected patients after excluding of any GI-organic diseases (Table 2). Multivariate ordered logistic regression analysis adjusted for age, sex, and PPI revealed that the symptom scores for hunger cramps, nausea, early satiety, belching, dysphagia, or odynophagia in HIV-infected patients were significantly higher than those in non-HIV-infected patients (Table 2).

In HIV-infected patients, we analyzed the effect of low CD4 counts on the severity of upper GI symptoms after excluding of any GI-organic diseases (n = 290). Multivariate ordered logistic regression analysis adjusted for age, sex, and PPI revealed that the symptom scores for early satiety was marginally associated with low CD4 cell count (<200 μL), but other symptoms were not (Table 3).

Predictive Symptoms of CE in HIV-infected and non-HIV-infected Patients

There were 211 patients with CE and 3936 patients without any organic GI disease (n = 4147). In multivariate analysis, none of symptoms was positively associated with CE in HIV-infected patients, whereas dysphagia and odynophagia were positively associated with CE in non-HIV-infected patients (Table 4). The other 7 symptoms were not associated with CE (Table 4). Individuals were classified as having positive upper GI symptoms if they scored ≥2 on the modified GSRS. The abovementioned symptoms remained associated with CE in both univariate and multivariate analyses (Supplementary Table 1).

Predictive Symptoms of Erosive Esophagitis in HIV-infected and non-HIV-infected Patients

There were 651 patients with EE and 3863 patients without any organic GI disease (n = 4514). In multivariate analysis, heartburn and acid regurgitation were positively associated with EE in HIV-infected patients, whereas heartburn, acid regurgitation, nausea, belching, dysphagia, and odynophagia were positively associated with EE in non-HIV-infected patients (Table 5). The other 3 symptoms were not associated with EE (Table 5). Individuals were classified as having positive upper GI symptoms if they scored ≥2 in the modified GSRS. The abovementioned symptoms remained associated with EE in both univariate and multivariate analyses (Supplementary Table 2).

Internal Consistency of Symptom Measurement

The test of internal consistency using Cronbach's α revealed that measurement of GI symptom scores with the 9 items were reliable for both HIV-infected patients (α, 0.86) and non-HIV-infected patients (α, 0.85) after excluding organic disease.

TABLE 2. Effect of HIV Infection on the Severity of Upper GI Symptoms After Excluding GI-Organic Diseases (n = 4001)

Upper GI Symptoms	Crude OR	*P* Value	Adjusted OR*	*P* Value
Epigastric pain	1.06 (0.85–1.32)	0.622	0.95 (0.75–1.21)	0.685
Heartburn	1.31 (1.06–1.64)	0.014	1.18 (0.94–1.49)	0.158
Acid regurgitation	1.24 (0.99–1.55)	0.067	1.16 (0.91–1.48)	0.229
Hunger cramps	1.55 (1.24–1.95)	<0.001	1.36 (1.07–1.73)	0.013
Nausea	1.85 (1.48–2.31)	<0.001	1.48 (1.17–1.89)	0.001
Early satiety	1.44 (1.13–1.82)	0.003	1.49 (1.16–1.92)	0.002
Belching	1.25 (0.99–1.56)	0.058	1.29 (1.02–1.65)	0.037
Dysphagia	1.14 (0.89–1.45)	0.300	1.33 (1.02–1.71)	0.032
Odynophagia	1.48 (1.13–1.94)	0.005	1.34 (1.00–1.80)	0.047

Note. Values in parentheses represent 95% confidence intervals.
GI = gastrointestinal, HIV = human immunodeficiency virus, OR = odds ratio.
*Adjusted for age, sex, and PPI use.

DISCUSSION

In this study, we investigated 9 upper GI symptoms using a validated 7-point Likert score and we diagnosed CE and EE—2 major esophageal benign diseases[10,19]—using endoscopy. First, multivariate analysis adjusted for age, sex, and PPI revealed that HIV-infected patients had higher upper GI symptom scores than non-HIV-infected patients even after excluding GI-organic diseases. Second, dysphagia and odynophagia were independently associated with CE in non-HIV-infected patients, whereas none of the upper GI symptoms were positively associated with CE in HIV-infected patients. Third, we found that heartburn and acid regurgitation were positively associated with EE in HIV-infected patients, whereas heartburn, acid regurgitation, nausea, belching, dysphagia, and odynophagia were positively associated with EE in non-HIV-infected patients.

The presence of HIV infection increased the severity of hunger cramps, nausea, early satiety, belching, dysphagia, and odynophagia. The reason for this remains unclear, but 1 possible explanation is upper GI dysmotility in HIV-infected patients. Konturek et al[20] found that HIV-infected patients with no morphologic changes in the upper GI tract on endoscopy had an abnormal gastric emptying rate, which suggests that upper GI dysmotility causes various upper GI symptoms in the absence of findings on endoscopy in HIV-infected patients. Another possible explanation is medication use. Nausea can occur with use of any protease inhibitor but is more common with zidovudine and didanosine among the nucleoside reverse transcriptase inhibitors.[21] Indinavir is also associated with esophageal reflux (~3%).[21] We did not record the medications of HAART because HIV-infected patients often use multiple medications and frequent changes are made to their medication regimens. In addition, our study showed no significant association between upper GI symptoms and low CD4 cell counts. One possible reason for this is that we used HAART drugs to HIV-infected patients whose CD4 cell count were relatively high according to the guideline.[22]

The information on upper GI symptoms predictive of CE is scarce. Some case series or retrospective studies showed an association between dysphagia or odynophagia and CE in non-HIV-infected patients.[10,23] Baehr et al[10] conducted a meta-analysis of 57 reports and found that 63% of CE patients presented with dysphagia or odynophagia and that these were the most frequent symptoms of CE among oral lesions, nausea/vomiting, abdominal pain, weight loss, fever, cough, diarrhea, and rash. Yakoob et al[23] conducted a retrospective endoscopy-based study and found that prevalence of retrosternal discomfort, dysphagia, and epigastric symptoms was 39.3%, 25.4%, and 35.3% in CE patients, compared with 30.3%, 19.7%, and 50% in non-CE patients, respectively. Although previous studies were small in scale, retrospective, did not use the validated

TABLE 3. Effect of Low CD4 (<200/μL) Counts on the Severity of Upper GI Symptoms Among HIV-Infected Patients After Excluding GI-Organic Diseases (n = 290)

Upper GI Symptoms	Crude OR	*P* Value	Adjusted OR*	*P* Value
Epigastric pain	0.94 (0.54–1.65)	0.840	0.77 (0.44–1.38)	0.385
Heartburn	0.76 (0.43–1.34)	0.342	0.67 (0.38–1.21)	0.183
Acid regurgitation	0.88 (0.49–1.55)	0.650	0.76 (0.42–1.37)	0.361
Hunger cramps	1.20 (0.69–2.08)	0.523	1.04 (0.59–1.83)	0.891
Nausea	1.69 (0.98–2.92)	0.057	1.39 (0.80–2.42)	0.245
Early satiety	1.70 (0.96–3.03)	0.069	1.64 (0.92–2.93)	0.095
Belching	0.97 (0.54–1.74)	0.923	0.93 (0.52–1.69)	0.823
Dysphagia	1.27 (0.70–2.31)	0.430	1.27 (0.70–2.33)	0.432
Odynophagia	1.49 (0.77–2.86)	0.237	1.38 (0.71–2.68)	0.348

Values in parentheses represent 95% confidence intervals.
HIV = human immunodeficiency virus, GI = gastrointestinal, OR = odds ratio.
*Adjusted for age, sex, and PPI use.

TABLE 4. Upper GI Symptom Scores for *Candida* Esophagitis in HIV-Infected and Non-HIV-Infected Patients (n = 4147)

HIV-Infected Patients (n = 320)				
Upper GI Symptom	**Crude OR**	***P* Value**	**Adjusted OR***	***P* Value**
Epigastric pain	0.74 (0.53–1.04)	0.080	0.72 (0.51–1.01)	0.058
Heartburn	0.80 (0.58–1.10)	0.174	0.79 (0.57–1.09)	0.153
Acid regurgitation	0.76 (0.53–1.10)	0.147	0.76 (0.52–1.09)	0.136
Hunger cramps	0.61 (0.39–0.94)	0.024	0.58 (0.37–0.90)	0.014
Nausea	0.52 (0.31–0.84)	0.008	0.47 (0.28–0.78)	0.004
Early satiety	1.00 (0.75–1.34)	0.996	1.00 (0.75–1.35)	0.975
Belching	0.95 (0.66–1.38)	0.800	0.97 (0.67–1.40)	0.872
Dysphagia	1.22 (0.92–1.62)	0.169	1.24 (0.93–1.64)	0.145
Odynophagia	1.18 (0.86–1.63)	0.310	1.18 (0.85–1.64)	0.318
Non-HIV-Infected Patients (n = 3827)				
Upper GI Symptoms	**Crude OR**	***P* Value**	**Adjusted OR**	***P* Value**
Epigastric pain	0.99 (0.87–1.12)	0.840	1.01 (0.88–1.15)	0.910
Heartburn	1.09 (0.95–1.24)	0.204	1.10 (0.96–1.26)	0.179
Acid regurgitation	1.06 (0.92–1.21)	0.439	1.05 (0.91–1.21)	0.529
Hunger cramps	0.96 (0.81–1.13)	0.630	0.97 (0.82–1.16)	0.759
Nausea	0.97 (0.84–1.13)	0.742	0.99 (0.85–1.16)	0.924
Early satiety	1.09 (0.92–1.30)	0.308	1.08 (0.90–1.29)	0.399
Belching	1.13 (0.97–1.32)	0.106	1.12 (0.96–1.31)	0.149
Dysphagia	1.24 (1.08–1.42)	0.002	1.20 (1.05–1.38)	0.008
Odynophagia	1.24 (1.03–1.50)	0.022	1.22 (1.01–1.48)	0.036

Values in parentheses represent 95% confidence intervals.
GI = gastrointestinal, HIV = human immunodeficiency virus, OR = odds ratio.
* Adjusted for age, sex, and PPI use.

TABLE 5. Upper GI Symptom Scores for Erosive Esophagitis in HIV-Infected and Non-HIV-Infected Patients (n = 4514)

HIV-Infected Patients (n = 333)				
Upper GI Symptom	**Crude OR**	***P* Value**	**Adjusted OR***	***P* Value**
Epigastric pain	1.06 (0.87–1.29)	0.554	1.12 (0.91–1.37)	0.289
Heartburn	1.34 (1.10–1.64)	0.004	1.37 (1.12–1.68)	0.003
Acid regurgitation	1.28 (1.05–1.57)	0.016	1.33 (1.08–1.64)	0.007
Hunger cramps	0.90 (0.69–1.16)	0.408	0.94 (0.72–1.22)	0.631
Nausea	0.88 (0.69–1.28)	0.317	0.91 (0.71–1.17)	0.453
Early satiety	0.77 (0.55–1.07)	0.120	0.80 (0.58–1.12)	0.202
Belching	1.03 (0.77–1.38)	0.826	1.07 (0.80–1.43)	0.637
Dysphagia	1.04 (0.77–1.39)	0.818	1.05 (0.78–1.40)	0.764
Odynophagia	0.71 (0.41–1.21)	0.209	0.74 (0.43–1.28)	0.281
Non-HIV-Infected Patients (n = 4181)				
Upper GI Symptom	**Crude OR**	***P* Value**	**Adjusted OR**	***P* Value**
Epigastric pain	1.00 (0.94–1.07)	0.983	1.03 (0.96–1.10)	0.449
Heartburn	1.27 (1.20–1.36)	<0.001	1.33 (1.24–1.42)	<0.001
Acid regurgitation	1.35 (1.27–1.43)	<0.001	1.40 (1.31–1.49)	<0.001
Hunger cramps	1.02 (0.94–1.10)	0.682	1.05 (0.96–1.14)	0.310
Nausea	1.09 (1.02–1.17)	0.016	1.12 (1.04–1.21)	0.002
Early satiety	1.04 (0.94–1.14)	0.449	1.09 (0.99–1.20)	0.097
Belching	1.12 (1.03–1.21)	0.007	1.16 (1.07–1.26)	<0.001
Dysphagia	1.12 (1.04–1.21)	0.004	1.18 (1.09–1.28)	<0.001
Odynophagia	1.19 (1.08–1.33)	0.001	1.23 (1.10–1.37)	<0.001

Values in parentheses represent 95% confidential intervals.
GI = gastrointestinal, HIV = human immunodeficiency virus, OR = odds ratio.
* Adjusted for age, sex, and PPI use.

symptom scale to quantitatively assess symptom severity, or excluded organic GI disease, their results are supported by our findings that dysphagia and odynophagia are symptoms predictive of CE in non-HIV-infected patients. The reason why only dysphagia and odynophagia are predictive of CE remains unclear, but a possible explanation is impairment of secondary peristalsis of the esophageal mucosa. Secondary peristalsis is a pressure wave of the esophagus that is triggered by esophageal distention.[24] Schoeman et al showed that patients with non-obstructive dysphagia commonly had defective secondary peristalsis in response to esophageal distension with boluses of air and water.[25] In CE patients, *Candida* species cover the esophageal mucosa and cause inflammation of the esophageal mucosa, which may possibly decrease the sensitivity to distension-induced secondary peristalsis and esophageal motility, leading to dysphagia and odynophagia. We found that none of the upper GI symptoms were positively associated with CE in HIV-infected patients. There have been few studies investigating upper GI symptoms and the presence of organic disease on endoscopy in HIV-infected patients; in particular, not many were prospective in design or had used a validated symptom scoring system. Corley et al[1] investigated the association between 14 upper GI symptoms and the presence of organic disease on endoscopy in HIV-infected patients using a 5-point Likert scale in a questionnaire-based study, and found that none of the symptoms independently predicted upper GI disease, a finding which is supported by our results. Interestingly, they also found that the frequency and severity of upper GI symptoms had negative correlations with the presence of organic diseases. This study and our study suggested that HIV-infected patients have various symptoms without organic diseases. The reason why none of the upper GI symptoms predicted CE in HIV-infected patients remains unknown, but we hypothesize that the nonspecificity of the symptoms may make it difficult to correlate particular symptoms with specific endoscopic abnormalities.

Multivariate analysis showed that heartburn and acid regurgitation can predict EE in both HIV-infected and non-HIV infected patients. Several studies have investigated the association between upper GI symptoms and EE in non-HIV-infected patients.[9,11] Okamoto et al[9] found that heartburn (OR, 2.46), odynophagia (OR, 1.36), and acid regurgitation (OR, 1.20) were independently associated with EE by multivariate analysis. Locke et al[11] found that heartburn frequency, acid regurgitation, and dysphagia were associated with EE. Although these studies investigated only 3 or 4 upper GI symptoms and had not excluded organic GI disease, their findings are in agreement with ours for non-HIV-infected patients. However, symptoms predictive of EE in HIV-infected patients have not been examined, although EE was common in not only non-HIV-infected patients (10.7%) but also HIV-infected patients (12.1%) in our study. In the present era of HAART, the prevalence of opportunistic diseases has decreased while that of non-opportunistic diseases has increased.[4,5] Moreover, PPI is commonly used in the treatment of EE, which interacts with some medications in HAART.[26] Therefore, management of EE is becoming more important in HIV-infected patients.

It is controversial whether performing endoscopy for diagnosis is appropriate for patients who have dysphagia, odynophagia, heartburn, or acid regurgitation, which are predictive symptoms of esophagitis. The current guidelines recommend empiric therapy with antifungal drugs or PPI for patients who are initially suspected of having esophagitis.[27,28] Therefore, we believe that endoscopy is appropriate for patients whose symptoms do not improve after empirical treatment to rule out other etiologies or to investigate the severity of esophagitis.

This study had several strengths. First, this was a large, prospective endoscopy-based study that enabled us to evaluate upper GI symptoms with adjustment for cofounders and exclusion of organic GI disease on endoscopy. Second, we confirmed the internal consistency of 9 GI symptoms in both HIV-infected and non-HIV-infected patients and found that they were reliable items. Third, all patients underwent the HIV serological test before endoscopy. However, this study has limitations. First, we did not assess psychological factors, which are closely associated with functional dyspepsia. Second, this is not a population-based study so selection bias was present. Third, although no significant difference was observed in endoscopy indication or the prevalence of organic GI diseases between HIV-infected and non-HIV-infected patients, the comparison between the small number of patients in the HIV group (n = 430) and the large number of patients in the non-HIV group (n = 5581) may lead to statistical bias.

In conclusion, this large-scale, endoscopy-based, prospective study demonstrated that severity of heartburn, hunger cramps, nausea, early satiety, belching, dysphagia, and odynophagia in HIV-infected patients was significantly greater than those in non-HIV-infected patients. None of the upper GI symptoms predicted CE in HIV-infected patients, whereas dysphagia and odynophagia predicted CE in non-HIV-infected patients. Heartburn and acid regurgitation predicted EE in both HIV-infected and non-HIV infected patients.

ACKNOWLEDGMENTS

We wish to express our gratitude to Clinical Research Coordinators Hisae Kawashiro, Sawako Iijima, Yoko Tanigawa, Aiko Gotanda, and Yaeko Sawada for their help with data collection.

REFERENCES

1. Corley DA, Cello JP, Koch J. Evaluation of upper gastrointestinal tract symptoms in patients infected with HIV. *Am J Gastroenterol.* 1999;94:2890–289610.
2. Werneck-Silva AL, Prado IB. Dyspepsia in HIV-infected patients under highly active antiretroviral therapy. *J Gastroenterol Hepatol.* 2007;22:1712–171611.
3. Knill-Jones RP. Geographical differences in the prevalence of dyspepsia. *Scandinavian J Gastroenterol Suppl.* 1991;182:17–24.
4. Monkemuller KE, Call SA, Lazenby AJ, et al. Declining prevalence of opportunistic gastrointestinal disease in the era of combination antiretroviral therapy. *Am J Gastroenterol.* 2000;95:457–4622.
5. Wilcox CM, Saag MS. Gastrointestinal complications of HIV infection: changing priorities in the HAART era. *Gut.* 2008;57:861–8706.
6. Naito Y, Yoshikawa T, Oyamada H, et al. Esophageal candidiasis. *Gastroenterologia Japonica.* 1988;23:363–3704.
7. Enns R, Amar J, Whittaker S, et al. 3383 Upper gastrointestinal endoscopy in hiv patients: indications and diagnostic yield in an era of highly active antiretroviral therapy (HAART). *Gastrointest Endosc.* 2000;51:AB774.
8. DeVault KR, Castell DO. American College of Gastroenterology. Updated guidelines for the diagnosis and treatment of gastroesophageal reflux disease. *Am J Gastroenterol.* 2005;100:190–2001.

9. Okamoto K, Iwakiri R, Mori M, et al. Clinical symptoms in endoscopic reflux esophagitis: evaluation in 8031 adult subjects. *Dig Dis Sci.* 2003;48:2237–224112.
10. Baehr PH, McDonald GB. Esophageal infections: risk factors, presentation, diagnosis, and treatment. *Gastroenterology.* 1994;106:509–5322.
11. Locke GR, Zinsmeister AR, Talley NJ. Can symptoms predict endoscopic findings in GERD? *Gastrointest Endosc.* 2003;58:661–6705.
12. Nishimura S, Nagata N, Shimbo T, et al. Factors associated with esophageal candidiasis and its endoscopic severity in the era of antiretroviral therapy. *PloS One.* 2013;8:e582173.
13. Asayama N, Nagata N, Shimbo T, et al. Relationship between clinical factors and severity of esophageal candidiasis according to Kodsi's classification. *Dis Esophagus.* 2014;27:214–2193.
14. Svedlund J, Sjodin I, Dotevall G. GSRS—a clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. *Dig Dis Sci.* 1988;33:129–1342.
15. Talley NJ, Fullerton S, Junghard O, et al. Quality of life in patients with endoscopy-negative heartburn: reliability and sensitivity of disease-specific instruments. *Am J Gastroenterol.* 2001;96:1998–20047.
16. Lundell LR, Dent J, Bennett JR, et al. Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. *Gut.* 1999;45:172–1802.
17. Richardson P, Hawkey CJ, Stack WA. Proton pump inhibitors. Pharmacology and rationale for use in gastrointestinal disorders. *Drugs.* 1998;56:307–3353.
18. Hansen JM, Wildner-Christensen M, Schaffalitzky de Muckadell OB. Gastroesophageal reflux symptoms in a Danish population: a prospective follow-up analysis of symptoms, quality of life, and health-care use. *Am J Gastroenterol.* 2009;104:2394–240310.
19. Dent J, El-Serag HB, Wallander MA, et al. Epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut.* 2005;54:710–7175.
20. Konturek JW, Fischer H, van der Voort IR, et al. Disturbed gastric motor activity in patients with human immunodeficiency virus infection. *Scandinavian J Gastroenterol.* 1997;32:221–2253.
21. Carr A, Cooper DA. Adverse effects of antiretroviral therapy. *Lancet.* 2000;356:1423–14309239.
22. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. *Department of Health and Human Services.* 2015:E-1.
23. Yakoob J, Jafri W, Abid S, et al. Candida esophagitis: risk factors in non-HIV population in Pakistan. *World J Gastroenterol.* 2003;9:2328–233110.
24. Fleshler B, Hendrix TR, Kramer P, et al. The characteristics and similarity of primary and secondary peristalis in the esophagus. *J Clin Invest.* 1959;38:110–1161 Part 1.
25. Schoeman MN, Holloway RH. Secondary oesophageal peristalsis in patients with non-obstructive dysphagia. *Gut.* 1994;35:1523–152811.
26. McCabe SM, Smith PF, Ma Q, et al. Drug interactions between proton pump inhibitors and antiretroviral drugs. *Expert Opin Drug Metab Toxicol.* 2007;3:197–2072.
27. Benson CA, Kaplan JE, Masur H, et al. Treating opportunistic infections among HIV-infected adults and adolescents: recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association/Infectious Diseases Society of America. *MMWR Recomm Rep.* 2004;53:1–112.
28. Shaheen NJ, Weinberg DS, Denberg TD, et al. Upper endoscopy for gastroesophageal reflux disease: best practice advice from the clinical guidelines committee of the American College of Physicians. *Ann Intern Med.* 2012;157:808–816.

STEM CELLS TRANSLATIONAL MEDICINE®

TISSUE-SPECIFIC PROGENITOR AND STEM CELLS

Therapeutic Potential of Adipose-Derived SSEA-3-Positive Muse Cells for Treating Diabetic Skin Ulcers

KAHORI KINOSHITA,[a] SHINICHIRO KUNO,[a] HISAKO ISHIMINE,[b] NORIYUKI AOI,[a] KAZUHIDE MINEDA,[a] HARUNOSUKE KATO,[a] KENTARO DOI,[a] KOJI KANAYAMA,[a] JINGWEI FENG,[a] TAKANOBU MASHIKO,[a] AKIRA KURISAKI,[b] KOTARO YOSHIMURA[a]

Key Words. Adult stem cells • Tissue regeneration • Adipose • Cell culture • Stem cell transplantation • Endothelial cell • Hyaluronan • Mesenchymal stem cells

[a]Department of Plastic Surgery, University of Tokyo Graduate School of Medicine, Tokyo, Japan; [b]Research Center for Stem Cell Engineering, National Institute for Advanced Industrial Science and Technology, Ibaraki, Japan

Correspondence: Kotaro Yoshimura, M.D., Department of Plastic Surgery, University of Tokyo School of Medicine, 7-3-1, Hongo, Bunkyo-Ku, Tokyo 113-8655, Japan. Telephone: 81-3-5800-8948; E-Mail: kotaro-yoshimura@umin.ac.jp

Received August 31, 2014; accepted for publication November 17, 2014; first published online in SCTM *Express* January 5, 2015.

1066-5099/2015/$20.00/0

http://dx.doi.org/10.5966/sctm.2014-0181

ABSTRACT

Stage-specific embryonic antigen-3 (SSEA-3)-positive multipotent mesenchymal cells (multilineage differentiating stress-enduring [Muse] cells) were isolated from cultured human adipose tissue-derived stem/stromal cells (hASCs) and characterized, and their therapeutic potential for treating diabetic skin ulcers was evaluated. Cultured hASCs were separated using magnetic-activated cell sorting into positive and negative fractions, a SSEA-3$^+$ cell-enriched fraction (Muse-rich) and the remaining fraction (Muse-poor). Muse-rich hASCs showed upregulated and downregulated pluripotency and cell proliferation genes, respectively, compared with Muse-poor hASCs. These cells also released higher amounts of certain growth factors, particularly under hypoxic conditions, compared with Muse-poor cells. Skin ulcers were generated in severe combined immunodeficiency (SCID) mice with type 1 diabetes, which showed delayed wound healing compared with nondiabetic SCID mice. Treatment with Muse-rich cells significantly accelerated wound healing compared with treatment with Muse-poor cells. Transplanted cells were integrated into the regenerated dermis as vascular endothelial cells and other cells. However, they were not detected in the surrounding intact regions. Thus, the selected population of ASCs has greater therapeutic effects to accelerate impaired wound healing associated with type 1 diabetes. These cells can be achieved in large amounts with minimal morbidity and could be a practical tool for a variety of stem cell-depleted or ischemic conditions of various organs and tissues. STEM CELLS TRANSLATIONAL MEDICINE *2015;4:146–155*

INTRODUCTION

Bone marrow has been used for many years as a treatment of leukemia, and the mesenchymal stem cells (MSCs) contained in transplanted bone marrow have been shown to differentiate into tissue-specific cells in various organs [1–3]. Although previous results suggested that MSCs derived from bone marrow or skin contain a fraction of pluripotent or multipotent cells [1, 2, 4, 5], the multipotent cells were not well characterized, because they were considered very rare and lacked a specific surface marker. Kuroda et al. [6] reported a unique subset of human MSCs that can be efficiently isolated as cells double positive for mesenchymal marker CD105$^+$ and human pluripotent stem cell marker stage specific embryonic antigen (SSEA)-3$^+$ cells. They are also able to self-renew and differentiate into cells representative of all three germ layers, namely endodermal, ectodermal, and mesodermal cells from a single cell [6]. These cells were initially identified by applying stress to MSCs and were termed multilineage differentiating stress-enduring cells (Muse cells). Although Muse cells do not have high proliferative activity, they were reported to generate multiple cell types of the three germ layers without inducing unfavorable tumors [6]. Thus, Muse cells appear to be safer than other induced pluripotent or multipotent cells and might have better therapeutic potential than general (non-Muse) MSCs. Although Muse cells were not identified in animals other than a goat [7], they have been sparsely detected around vessels in various human mesodermal organs or tissues such as the skin and subcutis [8–10]. Muse cells were also reported to home to injured regions and spontaneously differentiate into tissue-specific functional cells in the damaged liver, skin, and skeletal muscle in animal models [6].

In the present report, we isolated SSEA-3$^+$ Muse cells from patient-derived human adipose tissue-derived stem/stromal cells (hASCs). We characterized the adipose-derived Muse cells and evaluated their therapeutic potential in treating diabetic refractory skin ulcers. Skin ulcers under ischemic conditions generally show

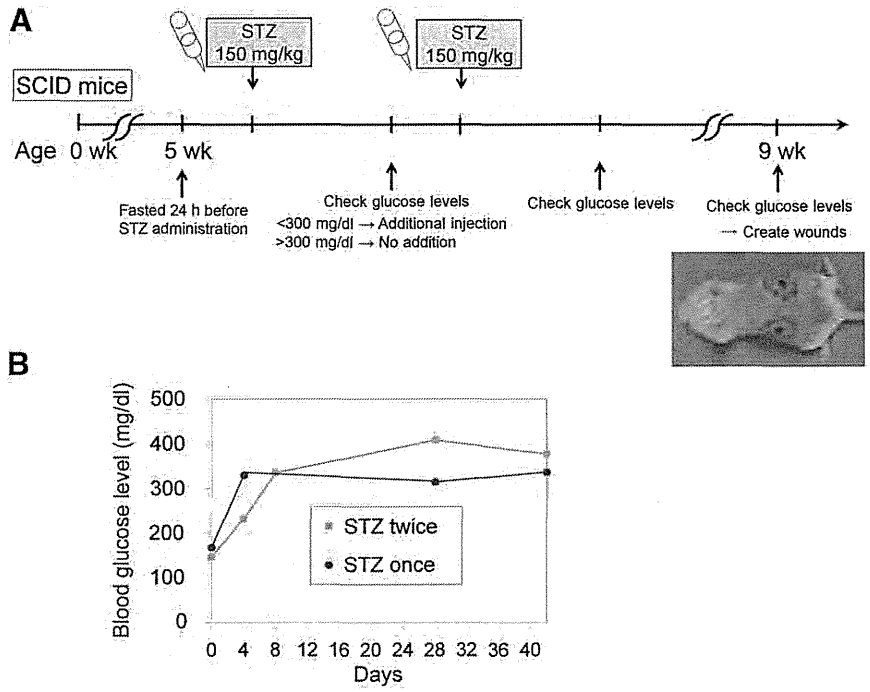

Figure 1. Preparation of immunodeficient diabetic mice. **(A):** We prepared immunodeficient mice with diabetes mellitus (DM) for a wound healing experiment. To induce type 1 DM, STZ was injected intraperitoneally into 5-week-old male SCID mice that had been fasted for 24 hours. Three days after STZ (150 mg/kg) administration, hyperglycemia (blood glucose >300 mg/dl) was examined. When hyperglycemia was not observed, another STZ (150 mg/kg) injection was given. Skin defects were created on the back of DM-induced SCID mice at 9 weeks of age. **(B):** Typical changes in blood glucose level are shown. Hyperglycemia was achieved after one or two STZ injections at approximately 75%. Abbreviations: SCID, severe combined immunodeficiency; STZ, streptozotocin.

delayed wound healing and are typical therapeutic targets of cell-based therapies using MSCs or endothelial progenitor cells. In order to investigate whether Muse cells are superior to other MSCs, we compared the wound healing of skin defects in diabetic mice between treatments with the two distinct cell populations.

Materials and Methods

Human Tissue Sampling and Cell Isolation

Liposuction aspirates were obtained from liposuction surgery from the abdomen and/or thighs after informed consent from 5 nonobese female patients (age 26.6 ± 8.7 years, body mass index 21.5 ± 2.0 kg/m^2). The ethical committee of the University of Tokyo School of Medicine approved the present study. The stromal vascular fraction (SVF) containing adipose-derived stem/stromal cells (ASCs) was isolated from the aspirated fat, as described previously [11]. In brief, aspirated fat tissue was washed with phosphate-buffered saline (PBS) and digested in PBS containing 0.075% collagenase for 30 minutes in a shaker at 37°C. Mature adipocytes and connective tissue were separated from pellets by centrifugation. The cell pellets were resuspended, filtered through 100-μm, 70-μm, and 40-μm mesh, and hemolyzed.

Cell pellets (equivalent to SVF) containing ASCs were cultured in dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. After approximately 2 weeks in culture, expanded hASCs were subcultured in the same media. Second-passage cultured hASCs were harvested with 0.25% trypsin containing 2 mM EDTA for 5 minutes at 37°C and used for Muse cell isolation.

Muse Cell Separation

Magnetic-activated cell sorting (MACS) (autoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) was used to collect SSEA-3$^+$ Muse cells. Although Muse cells are reported to be double positive for SSEA-3 and CD105, previous studies collected Muse cells using single SSEA-3 labeling because nearly 100% of mesenchymal cells, including ASCs, will be CD105$^+$ [6, 10, 12]. We, therefore, collected Muse cells using the anti-SSEA-3 antibody conjugated with phycoerythrin (PE) (dilution 1:3, Miltenyi Biotec) and anti-PE microbeads (dilution 1:2, Miltenyi Biotec) were used for MACS separation of the Muse cells. Target cell-labeled microbeads were trapped in a magnetic field and later collected as a positive fraction. The cell solution that did not attach to the magnetic column was collected as the negative fraction. To better purify a small number of Muse cells, we used a MACS program that applied the cell solution twice to the magnetic column at a very slow speed. The obtained positive cell fraction was considered the "Muse-rich" population, and the negative counterpart was considered the "Muse-poor" population.

Flow Cytometry Analyses

Flow cytometry analyses were performed before and after separation of the Muse cells using a flow cytometer (MACSQuant; Miltenyi Biotec). The SSEA-3 antibody conjugated with PE (dilution 1:3; Miltenyi Biotec) was used for the analyses.