Research Article

Initial viral load in cases of single human papillomavirus 16 or 52 persistent infection is associated with progression of later cytopathological findings in the uterine cervix

Daisuke Hamaguchi[1], Kiyonori Miura[1,*], Shuhei Abe[1], Akira Kinoshita[2], Shoko Miura[1], Kentaro Yamasaki[1], Koh-ichiro Yoshiura[2] and Hideaki Masuzaki[1]

Departments of [1]Obstetrics and Gynecology and [2]Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

*Correspondence: Dr. Kiyonori Miura, Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
Tel: +81-95-819-7363; Fax: +81-95-819-7365; E-mail: kiyonori@nagasaki-u.ac.jp

Running head: HPV DNA load and cytology in uterine cervix

The authors declare no conflict of interest

ABSTRACT

The aim of this study was to investigate the relationship between viral load in single human papillomavirus (HPV) 16 or 52 persistent infection and the progression of later cytopathological findings in the uterine cervix. Cervical cytology and HPV genotyping tests were repeated within 3–6 months in 305 women with oncogenic HPV. Twenty-four cases of single HPV 52 persistent infection and 24 cases of single HPV 16 persistent infection were identified. Cases with later cytopathological findings showing progression were defined as the progression group, while those with no change or regression were the non-progression group. Relative HPV DNA loads were determined by quantitative real-time polymerase chain reaction and expressed relative to human albumin (*ALB)* DNA. Differences between the two groups were evaluated. The median relative HPV 52 DNA load was 2.211 in the progression group and 0.022 in the non-progression group (Mann–Whitney U test, P=0.003). The median relative HPV 16 DNA load was 4.206 in the progression group and 0.103 in the non-progression group (P=0.001). HPV 52 and 16 DNA loads assessed by quantitative real-time methods may be useful short-term markers for identifying women at high risk for progression of cervical cytological pathology.

Keywords: cervical cytology / oncogenic human papillomavirus / persistent infection / progression / virus load

INTRODUCTION

Persistent infections with oncogenic human papillomaviruses (HPVs), including 16 HPV genotypes (16, 18, 31, 33, 35, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82) are recognized as a major risk factor for cervical cancer [Muñoz et al., 2003]. Genital HPV infections are common and are transmitted by sexual contact [Shimada et al., 2007]. However, most HPV infections disappear naturally over a relatively short period and are associated with little risk of developing disease [Moscicki et al., 1993; Ho et al., 1998; Woodman et al., 2001]. The presence of HPV per se therefore has a low predictive value for the risk of developing cervical cancer.

Screening of high-grade squamous intraepithelial lesion and cervical cancer are currently based on cervicovaginal pap smears with detection of oncogenic HPV [Inoue et al., 2010]. However, clinical interest in viral-load quantification has been demonstrated in human immunodeficiency virus and hepatitis B virus infections, where it is used routinely as a tool for diagnosis, prognosis, and therapeutic management [Berger and Preiser, 2002]. By contrast, the association between HPV viral load and evolution towards malignant cervical lesions remains debatable [Dalstein et al., 2003; Carcopino et al., 2006; Xi et al., 2009].

The HPV DNA load seems to predict the risk of developing cervical carcinoma prior to the appearance of any cytological alterations, and certainly long before the appearance of tumors [Ylitalo et al., 2000]. In particular, high HPV 16 loads in normal cervical smears are a risk marker for later development of cervical intraepithelial neoplasia or carcinoma in situ of the cervix [van Duin et al., 2002; Moberg et al., 2003]. In addition, high HPV 16 load is a risk marker for subsequent invasive cervical cancer [Moberg et al., 2005]. Testing for HPV 16 DNA load during gynecological health checks could thus strikingly improve our ability to distinguish between high- and low-risk infections in terms of progression to cervical cancer [Josefsson et al., 2000]. However, one study showed an increased odds ratio of prevalent

high-grade squamous intraepithelial lesion /cancer for HPV 16 load, but no similar trend for HPV 18 [Gravitt et al., 2003], while another study reported possible predictive values of viral loads in HPV 16- and 18-positive patients with low-grade squamous intraepithelial lesion cytology [Botezatu et al., 2009]. The clinical utility of viral-load testing for all oncogenic HPV genotypes therefore remains unclear.

HPV 16 and 18 account for approximately 70% of cancers and 50% of high-grade cervical intraepithelial neoplasia [Smith et al., 2007]. However, previous study showed that HPV 52 was a more common genotype in Nagasaki, Japan, compared with the distribution of high-risk HPV genotypes in other countries [Yamasaki et al., 2011a; Yamasaki et al., 2011b]. In addition, HPV 52 was the most common genotype among HPV-infected pregnant Japanese women. The second most common genotype was HPV 16, and these two genotypes collectively accounted for around 60% of HPV-positive pregnant women [Yamasaki et al., 2011b].

Another complication of using viral load to predict neoplasias of cervical intraepithelial neoplasia 2 or greater is the high prevalence of multiple oncogenic HPV infections detected in cervical samples. Recent studies have focused on longitudinal observations of viral load to predict viral clearance or lesion progression [Monnier-Benoit et al., 2006; Marks et al., 2011]. Initial data indicate that repeated measurements can improve prediction of persistence or clearance, but these data are currently limited to HPV 16. Because the screening of cervical disease is based on cervicovaginal pap smears with detection of oncogenic HPV, it is both important and necessary to clarify the association between the viral load of individual oncogenic HPV types in each region and the progression of later cytopathological findings in the uterine cervix.

In this study, the relationship between viral loads in single HPV 16 or 52 persistent infections and the progression of later cytopathological findings in the uterine cervix was

investigated to improve the understanding of the clinical utility of oncogenic HPV DNA loads in Japanese women.

MATERIALS AND METHODS

Study patients

A total of 305 women with oncogenic HPV underwent repeat cervical cytology and HPV DNA tests within 3–6 months, between August 2007 and April 2011. These 305 women included patients negative for intraepithelial lesion or malignancy, with atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesion or high-grade squamous intraepithelial lesion. Among these, 24 cases of single HPV 52 persistent infection and 24 cases of single HPV 16 persistent infection were included in this study, and the samples with multiple HPV types including HPV52 or HPV16 were excluded. The study protocol was approved by the Ethical Review Board of Nagasaki University and the other hospitals involved. All women were informed of the purpose of the study and gave their consent.

Sample collection and cytological diagnoses

Specimens were collected using a Cervex Brush (Rovers Medical Devices, Oss, the Netherlands) and suspended in 10 mL of SurePath preservative fluid (Becton, Dickinson & Company, Franklin Lakes, USA). Samples from the same vial for cytological testing with the Bethesda III system (2001) and for HPV genotype testing were used [Yamasaki et al., 2011a; Yamasaki et al., 2011b]. Cervical specimens for cytology and HPV genotyping were obtained at each visit from participants who received regular follow-up examinations. Cytologic diagnoses of the specimens were performed by the same experienced cytoscreener in a commercial laboratory (SRL, Tokyo, Japan), who was blinded to the results of the HPV

genotyping test. Regarding cervical cytopathological findings after 3–6 months, cases showing progression were defined as the progression group, and cases showing no change or regression were defined as the non-progression group.

Histopathological examinations

Colposcopies and biopsies were performed only when cervical cytopathological findings were detected. Histopathological diagnoses were made by two different pathologists, and cervical intraepithelial neoplasia is categorized according to World Health Organization Classification as follow: cervical intraepithelial neoplasia 1, 2 and 3 [Tavassoli and Devilee, 2003]. H&E photos of cervical intraepithelial neoplasia 1, 2 and 3 were shown in Figure 1.

HPV genotyping test

Genotyping of HPV DNA in SurePath preservative fluid was carried out after preparing glass slides, using the Linear Array HPV Genotyping Test Kit (Roche Molecular Systems, Indianapolis, USA), which uses PGMY09/PGMY11 primers [Gravitt et al., 2000] to amplify the L1 conserved region. Following polymerase chain reaction (PCR) amplification, hybridization of the HPV amplicon was performed using an array of oligonucleotide probes that allowed independent identification of individual HPV genotypes. This kit can detect the following 37 HPV genotypes: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39 and CP6108 (89). For consistency with previous studies, 16 HPV genotypes (16, 18, 31, 33, 35, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82) were considered as high-risk genotypes, which were related to cervical cancer in previous reports [Walboomers et al., 1999; Muñoz et al., 2003; Asato et al., 2004].

TaqMan probes and primers for HPV 52, HPV 16, and human albumin (ALB) gene

Primers and probes for HPV 52 and 16 quantification were located on the E7 gene and those for the *ALB* gene were on exon 12. Primers and probes were chosen using the Primer express program (Applied Biosystems, Warrington, UK). Probes were labeled with FAM^{TM}dye.

Measurement of relative HPV DNA loads by quantitative real-time PCR

Genomic DNA was extracted from cervical cell samples using a DNA Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA samples were prepared at 5 ng/μL using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). For each TaqMan assay, genomic DNA from a case with single HPV 52 persistent infection and from a case with single HPV 16 persistent infection were diluted in sterile water to obtain a calibration curve from 10^6 to 10 copies/μL of solution by 10-fold serial dilution. Real-time PCR was performed using the LightCycler480 (Roche Molecular Systems, Indianapolis, IN, USA). The primers and hybridization probe sequence for human albumin (*ALB*; GenBank accession no. M12523) were: forward primer: 5'-TTGGAAAAATCCCACTGCATT-3', reverse primer: 5'-GAAGGCAAGTCAGCAGGCAT-3', FAM reporter: 5'-CCGAAGTGGAAAATGATGA-3'. The equivalent sequences for HPV 52 E7 were: forward primer: 5'-CATTCATAGCACTGCGACGG-3', reverse primer: 5'-CTTGTAATGTGCCCAACAGCA-3', FAM reporter: 5'-CCTTCGTACTCTACAGCAA-3', and those for HPV 16 E7 were: forward primer: 5'-TCAGAGGAGGAGGATGAAATAGATG-3', reverse primer: 5'-AATGGGCTCTGTCCGGTTC-3', FAM reporter: 5'-CCAGCTGGACAAGC-3'. All PCR mixtures were prepared automatically using QIAgility (Qiagen, Hilden, Germany). Absolute quantitative real-time PCR (10 μL) were carried out in triplicate with 10 ng of unknown genomic DNA, 2× Quanti-Tect-Multiplex-PCR NoROX buffer (Qiagen, Hilden, Germany),

0.125 μM of each primer and 0.0625 μM of each TaqMan probe. Thermal cycling was initiated with a 2-min incubation at 50°C, followed by a first denaturation step of 15 min at 95°C, and then by 60 cycles of 15 sec at 95°C and 1 min at 60°C, followed by one cycle at 5°C for 6 min.

To adjust for differences in the amount of genomic DNA between samples, estimates of the amount of the nuclear gene *ALB* were made. Relative HPV DNA load was expressed as the number of HPV DNA copies relative to *ALB* DNA, which was considered to reflect the total human cellular DNA in the sample. The following formula was used: viral load = number of HPV copies/μL/number of *ALB* copies/μL.

Statistical analysis

Patient backgrounds were compared between the progression and non-progression groups using Student's *t*-tests and Fisher's exact tests for continuous and discrete variables, respectively. Regarding the relative HPV DNA loads, differences between the two groups were evaluated using Mann–Whitney U tests. Statistical analyses were performed with SPSS software version 19 (IBM Japan, Tokyo, Japan). Significant differences were defined as P values <0.05.

RESULTS

Characteristics of the progression and the non-progression groups in single HPV 16 or 52 persistent infection

Of the 24 cases of single HPV 52 persistent infection, eight were classified in the progression and the remaining sixteen in the non-progression group. Of the 24 cases with single HPV 16 persistent infection, 10 were classified in the progression and the remaining fourteen in the non-progression group. The characteristics of the progression and non-progression groups for

191 single HPV 16 and 52 persistent infections are described in Tables 1 and 2, respectively. Both
192 groups were similar in terms of age, interval between cervical cytological tests, parity and
193 body mass index. All study patients had engaged in sexual intercourse and were HIV
194 negative. The cytological findings were consistent with the results obtained from the
195 colposcopies and biopsies (Table 3 and Table 4).
196
197 **Relative HPV 52 DNA loads at first cytological sampling and changes in cervical**
198 **cytological findings**
199 Relative HPV DNA loads were described as the median (minimum-maximum). The median
200 relative HPV 52 DNA load in the progression group was significantly higher than in the non-
201 progression group (2.211; 0.088–13.089 versus 0.022; 0.001–0.618; Mann–Whitney U test,
202 *P*=0.003; Table 3 and Figure 2a).
203
204 **Relative HPV 16 DNA loads at first cytological sampling and changes in cervical**
205 **cytological findings**
206 Relative HPV DNA loads were described as the median (minimum-maximum). The median
207 relative HPV 16 DNA load in the progression group was significantly higher than in the non-
208 progression group (4.206: 0.407–38.999 versus 0.103; 0.001–96.566; Mann–Whitney U test,
209 *P*=0.001; Table 4 and Figure 2b).
210
211 **Relative HPV 52 and HPV 16 DNA loads in cervical cytological samples**
212 The prevalences of abnormal cytological findings (atypical squamous cells of undetermined
213 significance, low-grade squamous intraepithelial lesion and high-grade squamous
214 intraepithelial lesion) were similar in patients with single HPV 16 persistent infection and
215 those with HPV 52 persistent infection (Fisher's exact test, *P*=0.668). In addition, both the

relative HPV 52 and the relative HPV 16 DNA loads at entry were similar in cases with NILM and those with abnormal cytological findings (Mann–Whitney U test, $P>0.05$, respectively). However, the median relative HPV 16 DNA load in cases with single HPV 16 persistent infection was 0.636 (0.001–96.566), while the median relative HPV 52 DNA load in cases with single HPV 52 persistent infection was 0.065 (0.001–13.089) (Figure 3), indicating that the relative HPV 52 DNA load was significantly lower than the HPV 16 DNA load in the cervix in patients with single HPV infections (Mann–Whitney U test, $P=0.019$).

DISCUSSION

This preliminary study demonstrated a relationship between a high viral load in single HPV 16 persistent infection and the progress of cervical cytopathological findings, as observed previously [Lo et al., 2005; Carcopino et al., 2006], and also found a similar relationship for single HPV 52 persistent infection. The relative HPV 52 DNA load was significantly lower than the HPV 16 DNA load in cervical cells with single oncogenic HPV infection.

These results suggest that the initial viral load in cases with single HPV 52 or 16 persistent infection might be a predictive marker of later progression of cytopathological findings in the uterine cervix. The importance of the immune reaction in HPV infection is supported by the association of persistent infection with an increased risk of cervical cancer [Ho et al., 1998], and the amount of oncogenic HPV DNA may also reflect inherent differences between individuals in terms of their response to HPV 52 or 16 persistent infection. Systematic detection and quantification of oncogenic HPV DNA is also important not only for the screening of infection, but also for post-therapeutic follow-up [Carcopino et al., 2006], and high HPV DNA loads may have a critical value in predicting the evolution toward cytological abnormalities in women with no detectable cytological abnormalities in the uterine cervix [Carcopino et al., 2006]. However, the estimated positive predictive value

of HPV DNA load was too low for the test to be directly applicable as a single test for predicting cancer risk, except in women with the highest levels of HPV DNA [Josefsson et al., 2000]. Testing for oncogenic HPV DNA levels may thus be a useful addition to cytological screening for identifying women at high risk [Sun et al., 2002].

Persistent infection with oncogenic HPV types was found to be a key prognostic variable for risk of cytopathological progression [Moscicki et al., 2001; Schlecht et al., 2001; Dalstein et al., 2003], and the risk of cervical intraepithelial neoplasia 2/3 was increased in line with high initial oncogenic HPV loads. In this study, the influence of sexual intercourse and HIV infection on progression was identical between progression and non- progression groups (Table 1 and Table 2). The results demonstrate that the DNA load of single oncogenic HPV 52 or 16 infection may have a significant influence on the progression of cytopathological findings. Although higher viral loads may result from an increased rate of viral replication and may sustain viral persistence [Yoshida et al., 2008], this study demonstrated that the viral loads of HPV 52 and 16 at entry were not associated with the degree of cytologic abnormalities, supporting the idea that HPV DNA load seems to predict the risk of developing cervical carcinoma before any cytological alterations are visible [Ylitalo et al., 2000]. This result is not in accordance with previous studies [Carcopino et al., 2012; Al-Awadhi et al., 2013], and this discrepancy may reflect the small sample size used in the current study. Further studies using a greater number of patients are required to establish the reason for this discrepancy. It is interesting and necessary to monitor these women to identify any changes in cytology, HPV genotype or viral loads. Thus patients with normal cytology who present with high viral loads of HPV 52 or 16 should be monitored because of their risk of developing dysplastic lesions.

Multiple oncogenic HPV types are more common in cases of normal, atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesion and high-grade squamous intraepithelial lesion than in cases of invasive cervical carcinoma

[Yamasaki et al., 2011a; Yamasaki et al., 2011b]. To exclude the possibility of a high virus load being the result of infection with multiple HPV types, the current study estimated viral loads only in cases with single HPV 52 or 16 persistent infections, and samples with multiple HPV types including HPV 52 or HPV 16 were excluded. As in a previous study that found lower viral loads for HPV 18 (7.93×10^4 copies/μL) compared with HPV 16 (5×10^{13} copies/μL) [Botezatu et al., 2009], the results of this study also showed that the relative HPV 52 DNA load was significantly lower than the HPV 16 DNA load in the cervix in single HPV infections. In cases of multiple oncogenic HPV infections including HPV 16, it is difficult to estimate the risk of cervical cancer using oncogenic HPV DNA load itself because the viral load of HPV 16 may mask the influence of other oncogenic HPVs. For many other oncogenic HPV types, the causal attribution regarding the progression of later cytopathological findings is less clear, and future studies using multiple parallel evaluations of viral loads will be needed to understand the role of viral load in other carcinogenic types [Wentzensen et al., 2012]. The values of the viral load for samples collected 3 months later were not shown in this study. Future studies may investigate changes in HPV viral loads between initial and 3 months in an effort to identify factors which may affect progression of cytological findings.

In conclusion, the results of this study confirmed the key roles of HPV 52 and 16 DNA load in the progression of cytopathological findings. HPV 52 and 16 DNA loads measured by real-time quantitative methods may be useful as short-term markers for identifying women at high risk for progression of cervical cytopathology.

ACKNOWLEDGMENTS

This work was supported by the Japan Society for the Promotion of Science KAKENHI grant numbers nos. 23592406, 24791712 and 22591827. The authors would like to thank Hisayoshi Nakajima, Tetsuro Samejima, Akira Fujishita, Daisuke Nakayama, Kohei Kotera, Ai

291 Higashijima, Ozora Jo, Yasuko Noguchi, Michiharu Kohno, Takashi Tsukiyama, Yuri
292 Hasegawa, and Atsushi Yoshida for their assistance in this study.
293

294 **REFERENCES**

295 Al-Awadhi R, Chehadeh W, Al-Jassar W, Al-Harmi J, Al-Saleh E, Kapila K. 2013. Viral
296 load of human papillomavirus in women with normal and abnormal cervical cytology in
297 Kuwait. J Infect Dev Ctries 7:130–136.

298 Asato T, Maehama T, Nagai Y, Kanazawa K, Uezato H, Kariya K. 2004. A large case-control
299 study of cervical cancer risk associated with human papillomavirus infection in Japan, by
300 nucleotide sequencing-based genotyping. J. Infect. Dis. 189: 1829–1832.

301 Berger A, Preiser W. 2002. Viral genome quantification as a tool for improving patient
302 management: the example of HIV, HBV, HCV and CMV. J Antimicrob Chemother
303 49:713–721.

304 Botezatu A, Socolov D, Goia CD, Iancu IV, Ungureanu C, Huică I, Anton G. 2009. The
305 relationship between HPV16 and HPV18 viral load and cervical lesions progression.
306 Roum. Arch. Microbiol. Immunol.68: 175–182.

307 Carcopino X, Henry M, Benmoura D, Fallabregues AS, Richet H, Boubli L, Tamalet C.
308 2006. Determination of HPV type 16 and 18 viral load in cervical smears of women
309 referred to colposcopy. J. Med. Virol. 78: 1131–1140.

310 Carcopino X, Henry M, Mancini J, Giusiano S, Boubli L, Olive D, Tamalet C. 2012. Two years
311 outcome of women infected with high risk HPV having normal colposcopy following low-
312 grade or equivocal cytological abnormalities: are HPV16 and 18 viral load clinically useful
313 predictive markers? J Med Virol 84:964–972.

314 Dalstein V, Riethmuller D, Prétet JL, Le Bail Carval K, Sautière JL, Carbillet JP, Kantelip B,
315 Schaal JP, Mougin C. 2003. Persistence and load of high-risk HPV are predictors for
316 development of high-grade cervical lesions: a longitudinal French cohort study. Int. J.
317 Cancer 106: 396–403.

318 Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlée F, Hildesheim A, Schiffman MH,
319 Scott DR, Apple RJ. 2000. Improved amplification of genital human papillomaviruses. J.
320 Clin. Microbiol. 38: 357–361.
321 Gravitt PE, Burk RD, Lorincz A, Herrero R, Hildesheim A, Sherman ME, Bratti MC,
322 Rodriguez AC, Helzlsouer KJ, Schiffman M. 2003. A comparison between real-time
323 polymerase chain reaction and hybrid capture 2 for human papillomavirus DNA
324 quantitation. Cancer Epidemiol. Biomarkers Prev. 12: 477–484.
325 Ho, G. Y., Bierman, R., Beardsley, L., Chang, C. J., Burk, R. D. 1998. Natural history of
326 cervicovaginal papillomavirus infection in young women. N. Engl. J. Med. 338: 423–
327 428.
328 Inoue M, Okamura M, Hashimoto S, Tango M, Ukita T. 2010. Adoption of HPV testing as an
329 adjunct to conventional cytology in cervical cancer screening in Japan. Int J Gynaecol
330 Obstet 111:110–114.
331 Josefsson AM, Magnusson PK, Ylitalo N, Sørensen P, Qwarforth-Tubbin P, Andersen PK,
332 Melbye M, Adami HO, Gyllensten UB. 2000. Viral load of human papillomavirus 16 as a
333 determinant for development of cervical carcinoma in situ: a nested case-control study.
334 Lancet 355: 2189–2193.
335 Lo, K. W., Yeung, S. W., Cheung, T. H., Siu, N. S., Kahn, T. & Wong, Y. F. 2005. Quantitative
336 analysis of human papillomavirus type 16 in cervical neoplasm: a study in Chinese
337 population. J. Clin. Virol. 34: 76–80.
338 Marks M, Gravitt PE, Utaipat U, Gupta SB, Liaw K, Kim E, Tadesse A, Phongnarisorn C,
339 Wootipoom V, Yuenyao P, Vipupinyo C, Rugpao S, Sriplienchan S, Celentano DD. 2011.
340 Kinetics of DNA load predict HPV 16 viral clearance. J. Clin. Virol. 51: 44–49.

341 Moberg, M., Gustavsson, I. & Gyllensten, U. 2003. Real-time PCR-based system for
342 simultaneous quantification of human papillomavirus types associated with high risk of
343 cervical cancer. J. Clin. Microbiol. 41: 3221–3228.
344 Moberg, M., Gustavsson, I., Wilander, E. & Gyllensten, U. 2005. High viral loads of human
345 papillomavirus predict risk of invasive cervical carcinoma. Br. J. Cancer 92: 891–894.
346 Monnier-Benoit, S., Dalstein, V., Riethmuller, D., Lalaoui, N., Mougin, C. & Prétet, J. L.
347 2006. Dynamics of HPV16 DNA load reflect the natural history of cervical HPV-
348 associated lesions. J. Clin. Virol. 35: 270–277.
349 Moscicki, A. B., Palefsky, J., Smith, G., Siboshski, S. & Schoolnik, G. 1993. Variability of
350 human papillomavirus DNA testing in a longitudinal cohort of young women. Obstet.
351 Gynecol. 82 (Pt 1): 578–585.
352 Moscicki AB, Hills N, Shiboski S, Powell K, Jay N, Hanson E, Miller S, Clayton L, Farhat S,
353 Broering J, Darragh T, Palefsky J. 2001. Risks for incident human papillomavirus
354 infection and low-grade squamous intraepithelial lesion development in young females.
355 JAMA 285: 2995–3002.
356 Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer
357 CJ. International Agency for Research on Cancer Multicenter Cervical Cancer Study
358 Group. 2003. Epidemiologic classification of human papillomavirus types associated
359 with cervical cancer. N. Engl. J. Med. 348: 518–527.
360 Schlecht NF, Kulaga S, Robitaille J, Ferreira S, Santos M, Miyamura RA, Duarte-Franco E,
361 Rohan TE, Ferenczy A, Villa LL, Franco EL. 2001. Persistent human papillomavirus
362 infection as a predictor of cervical intraepithelial neoplasia. JAMA 286: 3106–3114.
363 Shimada, T., Miyashita, M., Miura, S., Nakayama, D., Miura, K., Fukuda, M., Masuzaki H.
364 2007. Genital human papilloma virus infection in mentally-institutionalized virgins.
365 Gynecol. Oncol. 106: 488–489.

366 Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, Clifford GM. 2007. Human
367 papillomavirus type distribution in invasive cervical cancer and high-grade cervical
368 lesions: a meta-analysis update. Int. J. Cancer 121: 621–632.
369 Sun, C. A, Liu, J. F, Wu, D. M., Nieh, S., Yu, C. P. & Chu, T. Y. 2002. Viral load of high-risk
370 human papillomavirus in cervical squamous intraepithelial lesions. Int. J. Gynaecol.
371 Obstet. 76: 41–47.
372 Tavassoli FA and Devilee P. 2003. World Health Organization Classification of Tumours.
373 Pathology & Genetics of Tumours of the Breast and Female Genital Organs. Lyon:
374 International Agency for Research on Cancer (IARC) Press. 270p.
375 van Duin M, Snijders PJ, Schrijnemakers HF, Voorhorst FJ, Rozendaal L, Nobbenhuis MA,
376 van den Brule AJ, Verheijen RH, Helmerhorst TJ, Meijer CJ. 2002. Human
377 papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN
378 II/III and viral clearance. Int. J. Cancer 98: 590–595.
379 Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ,
380 Peto J, Meijer CJ, Muñoz N. 1999. Human papillomavirus is a necessary cause of
381 invasive cervical cancer worldwide. J. Pathol. 189: 12–19.
382 Wentzensen N, Gravitt PE, Long R, Schiffman M, Dunn ST, Carreon JD, Allen RA, Gunja
383 M, Zuna RE, Sherman ME, Gold MA, Walker JL, Wang SS. 2012. Human
384 papillomavirus load measured by Linear Array correlates with quantitative PCR in
385 cervical cytology specimens. J. Clin. Microbiol. 50: 1564–1570.
386 Woodman CB, Collins S, Winter H, Bailey A, Ellis J, Prior P, Yates M, Rollason TP, Young
387 LS. 2001. Natural history of cervical human papillomavirus infection in young women:
388 A longitudinal cohort study. Lancet 357: 1831–1836.

389 Xi LF, Koutsky LA, Castle PE, Wheeler CM, Galloway DA, Mao C, Ho J, Kiviat NB. 2009.
390 Human papillomavirus type 18 DNA load and 2-year cumulative diagnoses of cervical
391 intraepithelial neoplasia grades 2-3. J Natl Cancer Inst 101:153–161.
392 Yamasaki K, Miura K, Shimada T, Ikemoto R, Miura S, Murakami M, Sameshima T,
393 Fujishita A, Kotera K, Kinoshita A, Yoshiura K, Masuzaki H. 2011a. Pre-vaccination
394 epidemiology of human papillomavirus infections in Japanese women with abnormal
395 cytology. J. Obstet. Gynaecol. Res. 37: 1666–1670.
396 Yamasaki K, Miura K, Shimada T, Miura S, Abe S, Murakami M, Sameshima T, Fujishita A,
397 Kotera K, Kinoshita A, Yoshiura K, Masuzaki H. 2011b. Epidemiology of human
398 papillomavirus genotypes in pregnant Japanese women. J. Hum. Genet. 56: 313–315.
399 Ylitalo N, Sørensen P, Josefsson AM, Magnusson PK, Andersen PK, Pontén J, Adami HO,
400 Gyllensten UB, Melbye M. 2000. Consistent high viral load of human papillomavirus 16
401 and risk of cervical carcinoma in situ: a nested case-control study. Lancet 355: 2194–
402 2198.
403 Yoshida T, Sano T, Kanuma T, Owada N, Sakurai S, Fukuda T, Nakajima T. 2008.
404 Quantitative real-time polymerase chain reaction analysis of the type distribution, viral
405 load, and physical status of human papillomavirus in liquid-based cytology samples from
406 cervical lesions. Int. J. Gynecol. Cancer 18: 121–127.
407

Titles and legends to figures

Figure 1. Histopathological findings of cervical intraepithelial neoplasia in surface epithelium. a) cervical intraepithelial neoplasia 1; The upper two-thirds of epithelium show maturation., b) cervical intraepithelial neoplasia 2: Nuclear abnormalities are more striking than in cervical intraepithelial neoplasia 1. The upper third of the epithelium shows maturation., c) cervical intraepithelial neoplasia 3: Squamous epithelium entirely of atypical basaloid cells.

Figure 2. Relative HPV DNA loads at first cytological sampling and changes in cervical cytology. a) Relative HPV 52 DNA loads and changes in cervical cytology. b) Relative HPV 16 DNA loads and changes in cervical cytology. Vertical axis indicates relative HPV DNA loads expressed as $\log_{10}$. Relative HPV 52 and 16 DNA loads were both significantly higher in the progression group than in the non-progression group (Mann–Whitney U tests, P=0.003 and 0.001, respectively).

Figure 3. Relative HPV 52 and HPV 16 DNA loads in cervix cytological samples. The median relative HPV 16 DNA load in cases with single HPV 16 persistent infection was 0.636 (0.001–96.566), while the median relative HPV 52 DNA load in cases with single HPV 52 persistent infection was 0.065 (0.001–13.089). The relative HPV 52 DNA load was significantly lower than the HPV 16 DNA load in the cervix in cases with single HPV infection (Mann–Whitney U test, P=0.019).

Table 1 Backgrounds of patients with single HPV 52 persistent infection

Characteristics	Progression group (n=8)	Non-progression group (n=16)	*P* value
Age at first sampling (years)[a]	36.0 (14.5)	34.0 (7.0)	NS[b]
Interval between cytological tests (months)[a]	4.6 (1.2)	4.1 (1.2)	NS[b]
Parity			
Nulliparous	3	7	NS[b]
Parous	5	8	
Body mass index (kg/m^2)[a]	21.4 (3.5)	21.2 (3.7)	NS[b]
Sexual intercourse	8	16	NS[b]
HIV infection	0	0	NS[b]

[a]Mean (SD); [b]NS indicates no significant difference between two groups (*t*-test and Fisher's exact test comparisons for continuous and discrete variables, respectively, in progression and non-progression groups). Significant differences were defined as $P<0.05$.

437 **Table 2 Backgrounds of patients with single HPV 16 persistent infection**

Characteristics	Progression group (n=10)	Non-progression group (n=14)	*P* value
Age at first sampling (years)[a]	36.0 (10.5)	37.5 (12.6)	NS[b]
Interval between cytological tests (months)[a]	4.8 (1.6)	4.4 (1.5)	NS[b]
Parity			
Nulliparous	5	5	NS[b]
Parous	5	6	
Body mass index (kg/m^2)[a]	22.9 (3.2)	22.2 (3.1)	NS[b]
Sexual intercourse	10	14	NS[b]
HIV infection	0	0	NS[b]

438 [a]Mean (SD); [b]NS indicates no significant difference between two groups (*t*-test and Fisher's
439 exact test comparisons for continuous and discrete variables, respectively, in progression and
440 non-progression groups). Significant differences were defined as $P<0.05$.

441

442 **Table 3 HPV 52 DNA load and cervical cytopathological changes in cases of single HPV**

443 **52 persistent infection**

Cases	Relative HPV virus load (HPV DNA/ALB DNA) at first sampling	Cytological findings		Cytological change	Results of colposcopy or biopsies	
		First sampling	Second sampling		First sampling	Second sampling
1	0.08782	LSIL	HSIL	Progression	CIN2	CIN3
2	0.29985	NILM	ASC-US	Progression	Not tested	CIN1
3	0.43585	LSIL	HSIL	Progression	CIN2	CIN3
4	0.51325	NILM	LSIL	Progression	Not tested	CIN1
5	2.21097	NILM	LSIL	Progression	Not tested	CIN2
6	6.42910	NILM	LSIL	Progression	Not tested	CIN2
7	7.75832	LSIL	HSIL	Progression	CIN1	CIN3
8	13.0894	NILM	HSIL	Progression	Not tested	CIN2
9	0.00125	NILM	NILM	Non-progression	Not tested	Not tested
10	0.00162	NILM	NILM	Non-progression	Not tested	Not tested
11	0.00379	HSIL	LSIL	Non-progression	CIN2	CIN1
12	0.00586	LSIL	NILM	Non-progression	NCF	Not tested
13	0.00706	NILM	NILM	Non-progression	Not tested	Not tested
14	0.00840	NILM	NILM	Non-progression	Not tested	Not tested
15	0.01412	NILM	NILM	Non-progression	Not tested	Not tested
16	0.01667	ASC-US	NILM	Non-progression	NCF	Not tested
17	0.02227	NILM	NILM	Non-progression	Not tested	Not tested
18	0.03270	NILM	NILM	Non-progression	Not tested	Not tested
19	0.03931	NILM	NILM	Non-progression	Not	Not

					tested	tested
20	0.06276	LSIL	LSIL	Non-progression	CIN1	CIN1
21	0.06743	LSIL	NILM	Non-progression	CIN1	CIN1
22	0.07969	NILM	NILM	Non-progression	Not tested	Not tested
23	0.10207	LSIL	LSIL	Non-progression	CIN2	CIN2
24	0.61879	HSIL	ASC-US	Non-progression	CIN2	CIN1

444 NILM: negative for intraepithelial lesion or malignancy, ASC-US: atypical squamous cells of
445 undetermined significance, LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade
446 squamous intraepithelial lesion, CIN: cervical intraepithelial neoplasia, NCF: normal
447 colposcopic findings
448

449 **Table 4 HPV 16 DNA load and cervical cytopathological changes in cases of single HPV**

450 **16 persistent infection**

Cases	Relative HPV virus load (HPV DNA/ALB DNA) at the first sampling	Cytological findings		Cytological change	Results of colposcopy or biopsies	
		First sampling	Second sampling		First sampling	Second sampling
1	0.40754	NILM	HSIL	Progression	Not tested	CIN2
2	2.15193	LSIL	HSIL	Progression	CIN1	CIN3
3	2.21686	NILM	HSIL	Progression	Not tested	CIN3
4	4.08485	LSIL	HSIL	Progression	CIN1	CIN3
5	4.12118	ASC-US	HSIL	Progression	CIN1	CIN2
6	4.29190	ASC-US	LSIL	Progression	NCF	CIN1
7	6.70919	LSIL	HSIL	Progression	CIN1	CIN3
8	8.49491	ASC-US	HSIL	Progression	Chronic cervicitis	CIN3
9	22.40488	LSIL	HSIL	Progression	CIN2	CIN3
10	38.99907	ASC-US	LSIL	Progression	Chronic cervicitis	CIN2
11	0.00060	LSIL	LSIL	Non-progression	CIN1	Chronic cervicitis
12	0.01041	HSIL	HSIL	Non-progression	CIN3	CIN3
13	0.01485	LSIL	LSIL	Non-progression	CIN3	CIN3
14	0.02130	NILM	NILM	Non-progression	Not tested	Not tested
15	0.02540	HSIL	HSIL	Non-progression	CIN3	CIN3
16	0.70239	HSIL	HSIL	Non-progression	CIN3	CIN3
17	0.07359	HSIL	HSIL	Non-progression	CIN3	CIN3
18	0.13196	NILM	NILM	Non-progression	Not tested	Not tested
19	0.15679	NILM	NILM	Non-progression	Not tested	Not tested

20	0.40484	LSIL	LSIL	Non-progression	CIN2	CIN2
21	0.57038	HSIL	HSIL	Non-progression	CIN3	CIN3
22	0.07042	HSIL	HSIL	Non-progression	CIN3	CIN2
23	0.86522	HSIL	HSIL	Non-progression	CIN3	CIN3
24	96.56597	HSIL	HSIL	Non-progression	CIN3	CIN3

NILM: negative for intraepithelial lesion or malignancy, ASC-US: atypical squamous cells of undetermined significance, LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion, CIN: cervical intraepithelial neoplasia, NCF: normal colposcopic findings

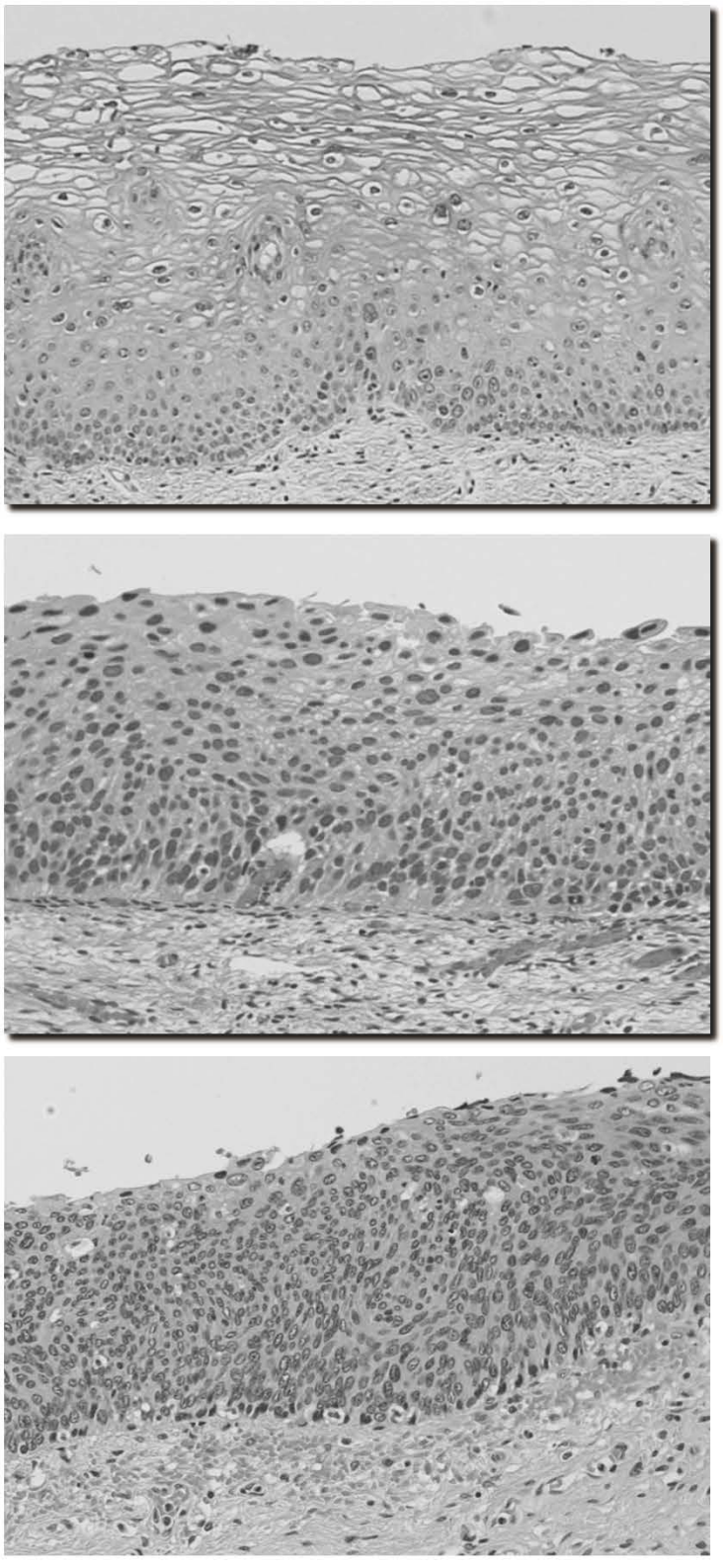

Figure 1. Histopathological findings of cervical intraepithelial neoplasia in surface epithelium. a) cervical intraepithelial neoplasia 1; The upper two-thirds of epithelium show maturation. There is mild atypia throughout., b) cervical intraepithelial neoplasia 2: Nuclear abnormalities are more striking than in cervical intraepithelial neoplasia 1. The upper third of the epithelium shows maturation., c) cervical intraepithelial neoplasia 3: Squamous epithelium entirely of atypical basaloid cells.

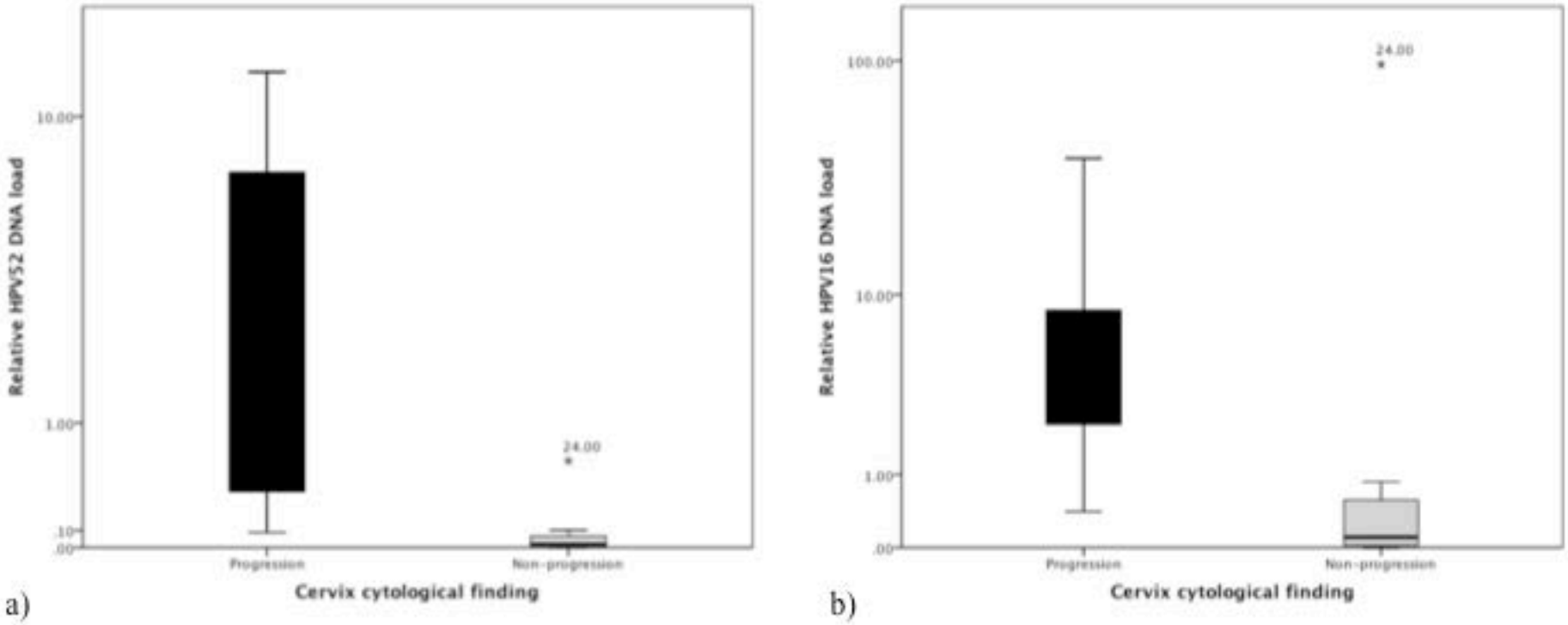

Figure 2 Relative HPV DNA loads at first cytological sampling and changes in cervical cytology.
a) Relative HPV 52 DNA loads and changes in cervical cytology. b) Relative HPV 16 DNA loads and changes in cervical cytology. Vertical axis indicates relative HPV DNA loads expressed as $\log_{10}$. Relative HPV 52 and 16 DNA loads were both significantly higher in the progression group than in the non-progression group (Mann-Whitney U tests, P=0.003 and 0.001, respectively).

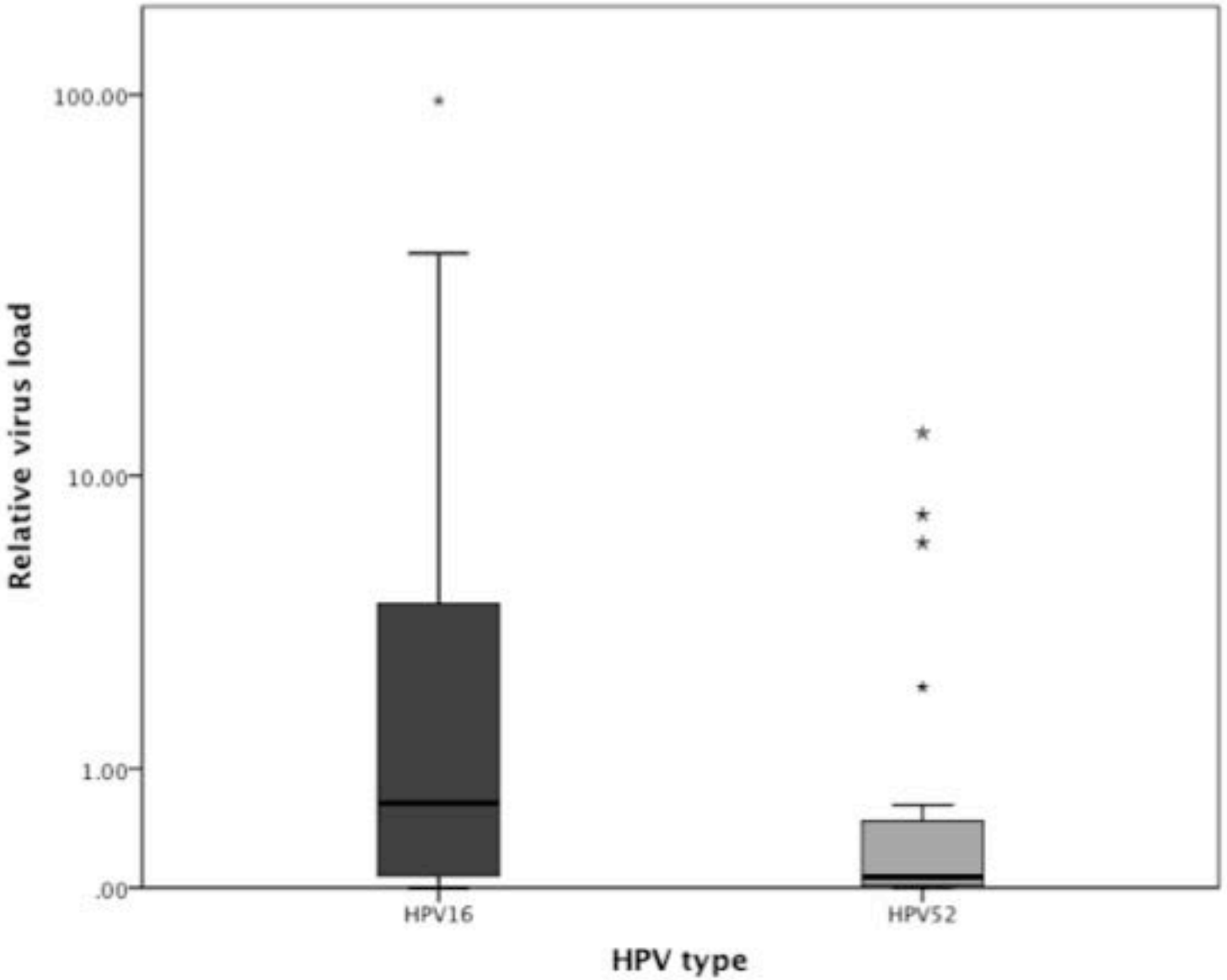

Figure 3 Relative HPV 52 and HPV 16 DNA loads in cervix cytological samples.
The median relative HPV 16 DNA load in cases with single HPV 16 persistent infection was 0.636 (0.001–96.566), while the median relative HPV 52 DNA load in cases with single HPV 52 persistent infection was 0.065 (0.001–13.089). The relative HPV 52 DNA load was significantly lower than the HPV 16 DNA load in the cervix in cases with single HPV infection (Mann-Whitney U test, P=0.019).