

Figure 1 Overall cumulative recurrence rate after treatment. The overall cumulative recurrence rate of cervical intraepithelial neoplasia 3 in the first 12 months after treatment was 22.6%. RFS, recurrence-free survival.

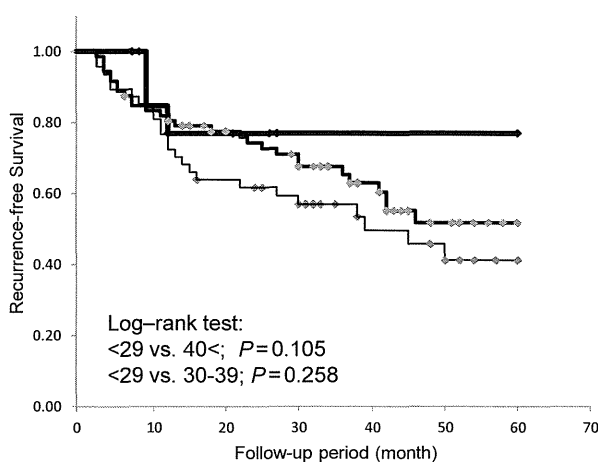


Figure 2 Recurrence rates compared by age. No significant elevated risk of recurrence was seen between younger age (<29) and older age (<29 vs >40; $P = 0.105$, <29 vs 30-39; $P = 0.258$, by log-rank test). —, <29; —, 30-39; —, >40.

(Fig. 1). Figure 2 shows recurrence rates compared by age. It is worth noting that no significant elevated risk of recurrence was seen between younger age (<29) and older age (<29 vs >40, $P = 0.105$; <29 vs 30-39, $P = 0.258$, by log-rank test). Among recurrence cases, 27 patients underwent second laser ablation, nine patients underwent cervical conization, and one patient underwent total hysterectomy. Other patients were followed up with intensive cytological and colposcopic evaluations

Table 1 Distribution of HPV type before treatment

| HPV type before treatment | Rate (%) |
|---------------------------|----------|
| 16 | 34 |
| 18 | 2 |
| 31 | 2 |
| 33 | 5 |
| 52 | 20 |
| 58 | 13 |
| Others | 24 |

The HPV genotypes of 83 patients were identified by polymerase chain reaction-based HPV DNA testing both before and after ablation. Seventy-six women were positive on HPV-DNA testing (91.6%). The distribution of HPV genotypes was classified according to the Matsumoto criteria of 8 'higher-risk' types. Two of the higher-risk types (35, 45) were not detected in this study. All cervical intraepithelial neoplasia 3 cases in this study had mono-infection. HPV, human papillomavirus.

with informed consent. The re-recurrence rate of CIN3 after second ablation was 11.1%.

Next, we evaluated the association between HPV genotype and efficacy of laser ablation, assessing whether HPV genotyping might predict failure after laser ablation. Of the 134 patients, we examined the HPV genotype of 83 patients, identified by PCR-based HPV DNA testing both before and after ablation. Single infection was observed in all CIN3 cases in this study, although there is a possibility that some patients might have multiple infections, especially in the CIN1-2 population. The median follow-up period was 17 months, with a minimum follow-up of 6 months. The median age of these 83 patients was 36 years; 76 women were positive on HPV-DNA testing (91.6%). The distribution of HPV genotypes, classified according to the Matsumoto criteria of eight 'higher-risk' types, before laser ablation is shown in Table 1.

Table 2 shows detection rates – that is, whether the same HPV type was detected before and after laser ablation, which can be interpreted as a good indicator of disease clearance. HPV was persistently detected after treatment more often in the higher-risk types, especially in type 16 and 18, than in other HPV types (16/18 vs 31/33/52/58 vs others; 66.6% vs 52.4% vs 25.0%; $P < 0.0001$ by Cochran-Armitage test).

In addition, we compared post-treatment RFS by HPV genotypes. As shown in Figure 3, we first compared RFS by pre-treatment HPV genotypes, that is, between those who were positive for the 8 higher-risk types and those positive for other HPV types before ablation; no statistically significant difference was seen between the two ($P = 0.77$ by log-rank test). We then

Table 2 Detection rate of HPV after treatment for each HPV type before treatment

| HPV type before treatment | Detection of HPV after treatment† | |
|---------------------------|-----------------------------------|--------------|
| | Positive (%) | Negative (%) |
| 16/18 | 66.6 | 33.3 |
| 31/33/52/58 | 52.4 | 47.6 |
| Others | 25.0 | 75.0 |

†Cochran–Armitage test $P < 0.0001$. Detection rates – i.e. whether the same HPV type was detected before and after laser ablation, which can be interpreted as a good indicator of disease clearance. HPV was persistently detected after treatment more often in the higher-risk types, especially in types 16 and 18, than in other HPV types (16/18 vs 31/33/52/58 vs others; 66.6% vs 52.4% vs 25.0%; $P < 0.0001$ by Cochran–Armitage test). HPV, human papillomavirus.

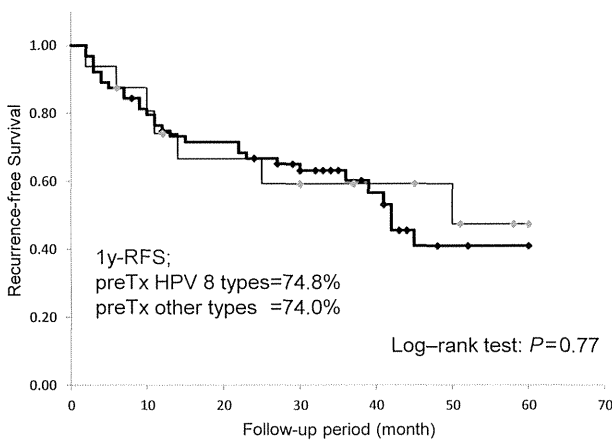


Figure 3 Recurrence-free survival (RFS) by pre-treatment human papillomavirus (HPV) genotype. We compared RFS between those who were positive for the 8 higher-risk types versus those positive for other HPV types prior to ablation; no significant difference between the two groups was identified. —, 8 types; - - -, Others.

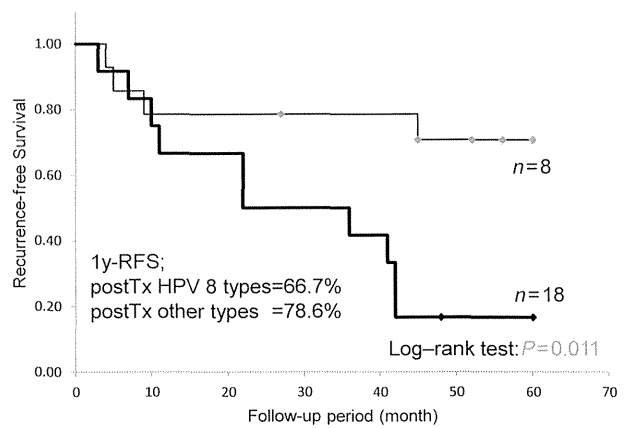


Figure 4 Recurrence-free survival (RFS) by post-treatment human papillomavirus (HPV) genotype. After ablation, 1-year RFS was 66.7% for those who were positive for the 8 higher-risk HPV types, which was significantly lower than the 78.6% observed in those positive for other HPV types ($P = 0.011$ by log-rank test). —, 8 types; - - -, Others.

compared RFS by post-treatment HPV genotypes. As shown in Figure 4, 1-year RFS was 66.7% for those who were positive for the 8 higher-risk types after ablation, which was significantly lower than the 78.6% observed in those positive for other HPV types ($P = 0.011$ by log-rank test). Furthermore, Figure 5 shows that the 1-year RFS for those positive for all high-risk 13 types of HPV after treatment was 61.1%, versus 100% in HPV-negative subjects ($P = 0.0013$ by log-rank test).

All these data suggest that the persistence of high-risk HPV genotypes, especially of the eight types, might play a key role in the development of recurrence.

Discussion

In 2011, a large Dutch multi-cohort study, one of the representative prospective studies on long-term

efficacy of CIN treatment, reported on recurrence risk after treatment for CIN2/3.¹⁶ According to that study, the recurrence risk of CIN was quite low after highly successful treatment followed by normal cytology or negative HPV testing. However, all participants were treated by excisional methods, such as cold-knife conization or LEEP, so the data did not present any information on the prognostic outcomes of conservative treatments, including laser ablation.

As mentioned, many studies have demonstrated that conservative methods, including cold-knife conization, LEEP, cryotherapy, and laser ablation, show similarly low morbidity and are equally successful at preventing invasive cervical cancer.^{4,17,18} However, in 2009, Melnikow *et al.*¹⁰ compared the long-term follow-up of 37 142 patients with CIN by treatment modality (cold-knife conization, LEEP, laser [conization and ablation

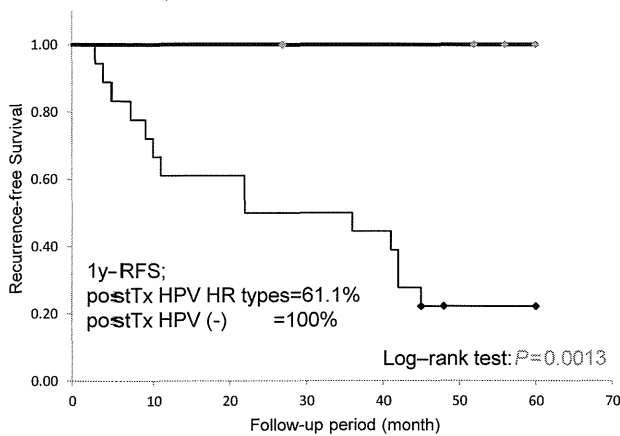


Figure 5 Recurrence-free survival (RFS) between post-treatment human papillomavirus (HPV) genotypes. Post-treatment 1-year RFS for those positive for 13 high-risk HPV was 61.1%, versus 100% for those who were HPV-negative. It is unsurprising that no recurrence was seen in any HPV-negative cases after laser ablation. —, negative; —, high-risk types.

combined], and cryotherapy), reporting that overall cumulative rate of CIN2/3 recurrence in the first 6 years after treatment of 14.0% for women originally treated for CIN3. Estimated rates of CIN2/3 after treatment were lowest for cold-knife conization, followed by LEEP and laser, and highest for cryotherapy. This result corroborates the invasiveness of the respective procedure.

Further evidences are warranted for the treatments of CIN3 though; considering many women with high-grade CIN are of reproductive age, treatment should not only be effective but also have minimum adverse effects on future fertility and obstetric outcomes. Several previous studies have clearly demonstrated that excisional treatments result in long-term adverse obstetric outcomes, including preterm delivery, low birthweight, or premature rupture of membranes; thus, an increasing number of patients are likely to choose less invasive treatment like laser ablation.⁵

The data in our study suggest that laser ablation is an optional conservative treatment for CIN3 with minimal invasiveness. Regarding obstetric outcomes, it is difficult to follow patients after CIN treatment in terms of pregnancy because of the premalignant nature of the lesion, so no data were available in this study. Nevertheless, as laser ablation has thus far been shown to be free of any adverse effect on obstetric outcomes, it should be taken into consideration especially for young patients of child-bearing age. Even for lesions, provi-

sional use of laser ablation may provide a 'grace period' for young women with desire to bear children before more invasive interventions are performed. Therefore, many women can postpone, or even entirely avoid, the unnecessary adverse outcomes associated with invasive intervention.

With regard to follow-up surveillance after treatment of high-grade CIN, the role of HPV-DNA testing has been an area of interest due to its high sensitivity and negative predictive value (NPV) for detecting recurrent disease. In a meta-analysis of 11 studies by Zielinski *et al.*,¹² sensitivity, NPV, and specificity of high-risk HPV testing for recurrence were 91% (95% CI = 86–95), 98% (97–99), and 79% (76–82), respectively. That study demonstrated that combining cytology with high-risk HPV testing increases rates even further: sensitivity was 96% (89–99), NPV was 99% (98–100), and specificity was 81% (77–84). Specificity is indeed relatively low, but considering that this is not a screening test but rather a follow-up for a potentially lethal disease, NPV and sensitivity should be valued higher than specificity.

We demonstrated in this study that HPV infection is not likely to disappear, but persists after laser ablation in women positive for eight 'higher-risk' HPV. Considering that cervical cancer is caused by persistent infection with a subset of carcinogenic HPV, this failure of viral clearance should lead to the increase in cervical cancer in future. Indeed, we confirmed the significant difference in RFS between those who were positive for the eight higher-risk types of post-treatment versus those who were positive for other types or altogether negative. This result supports the efficacy of HPV testing and is consistent with several previous studies.

However, several critical problems with HPV-PCR testing remain. First, while PCR testing can distinguish individual genotype, detection sensitivities differ greatly among HPV genotypes. Furthermore, routine PCR-based HPV DNA testing is not suitable for daily clinical use due to its high cost and complex technique using specific primers. For the purposes of this study context, therefore, we recommend commercially available HPV testing using the hybrid capture method, Hybrid Capture II (HC2), which targets a group of 13 high-risk HPV genotypes but does not distinguish which genotypes are present. This intensive test also represents well the eight higher-risk genotypes; identification of the existence of high-risk HPV by HC2 may sufficiently inform the likelihood of recurrence. In Japan, HC2 has been covered by insurance since 2010, but the target of this test is only limited for patients

with atypical squamous cells of undetermined significance (ASC-US) detected by screening cytology test; we recommend that HC2 should also be used widely for the surveillance of patients after treatment for CIN.

In summary, laser ablation is a useful conservative treatment for CIN3, especially for young women of reproductive age. It will continue to be necessary for gynecologists to tailor treatment strategies for young patients according to their health needs, balancing minimal adverse obstetric outcomes with minimal attenuation of efficacy. In addition, it is well established that the rate of developing high-grade disease, including invasive cervical cancer, remains elevated in these post-treatment patients, even after a very long time subsequent to treatment. Indeed, a study in 2006 warns that the rate of invasive disease remained at 56 per 100 000 woman-years, 2.8 times greater than expected, for at least 20 years after treatment.¹⁹ Because of this substantial long-term risk, close monitoring remains critical, and combined cytological evaluation together with HPV testing should increase the safety of the surveillance. For more rigorous and detailed follow-up algorithms, including surveillance intervals, obstetric outcomes, and cost-effectiveness, larger and longer-term studies are warranted in the future.

Acknowledgments

The authors are grateful to Dr Gautam A. Deshpande (St. Luke's International Hospital) for the critical reading of our manuscript.

Disclosure

The authors have no conflicts of interest related to this article.

References

- Herbert A, Smith JA. Cervical intraepithelial neoplasia grade III (CIN III) and invasive cervical carcinoma: The yawning gap revisited and the treatment of risk. *Cytopathology* 1999; **10**: 161–170.
- Bevis KS, Biggio JR. Cervical conization and the risk of preterm delivery. *Am J Obstet Gynecol* 2011; **205**: 19–27.
- Burke L, Covell L, Antonioli D. Carbon dioxide laser therapy of cervical intraepithelial neoplasia: Factors determining success rate. *Lasers Surg Med* 1980; **1**: 113–122.
- Chirenje ZM, Rusakaniko S, Akino V *et al.* A randomized clinical trial of loop electrosurgical excision procedure (LEEP) versus cryotherapy in the treatment of cervical intraepithelial neoplasia. *J Obstet Gynaecol* 2001; **21**: 617–621.
- Kyrgiou M, Koliopoulos G, Martin-Hirsch P *et al.* Obstetric outcomes after conservative treatment for intraepithelial or early invasive cervical lesions: Systematic review and meta-analysis. *Lancet* 2006; **367**: 489–498.
- Arbyn M, Kyrgiou M, Simoons C *et al.* Perinatal mortality and other severe adverse pregnancy outcomes associated with treatment of cervical intraepithelial neoplasia: Meta-analysis. *BMJ* 2008; **337**: a1284.
- Raju KS, Henderson E, Trehan A *et al.* A study comparing LETZ and CO2 laser treatment for cervical intra epithelial neoplasia with and without associated human papilloma virus. *Eur J Gynaecol Oncol* 1995; **16**: 92–96.
- Gonzalez DI Jr, Zahn CM, Retzlaff MG *et al.* Recurrence of dysplasia after loop electrosurgical excision procedures with long-term follow-up. *Am J Obstet Gynecol* 2001; **184**: 315–321.
- Dey P, Gibbs A, Arnold DF *et al.* Loop diathermy excision compared with cervical laser vaporisation for the treatment of intraepithelial neoplasia: A randomised controlled trial. *BJOG* 2002; **109**: 381–385.
- Melnikow J, McGahan C, Sawaya GE *et al.* Cervical intraepithelial neoplasia outcomes after treatment: Long-term follow-up from the British Columbia Cohort Study. *J Natl Cancer Inst* 2009; **101**: 721–728.
- Muñoz N, Bosch FX, de Sanjose S *et al.* International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003; **348**: 518–527.
- Zielinski GD, Bais AG, Helmerhorst TJ *et al.* HPV testing and monitoring of women after treatment of CIN 3: Review of the literature and meta-analysis. *Obstet Gynecol Surv* 2004; **59**: 543–553.
- Wright TC Jr, Massad LS, Dunton CJ *et al.* 2006 American Society for Colposcopy and Cervical Pathology-sponsored Consensus Conference. 2006 consensus guidelines for the management of women with abnormal cervical cancer screening tests. *Am J Obstet Gynecol* 2007; **197**: 346–355.
- Matsumoto K, Oki A, Furuta R *et al.* Japan HPV and Cervical Cancer Study Group. Predicting the progression of cervical precursor lesions by human papillomavirus genotyping: A prospective cohort study. *Int J Cancer* 2011; **128**: 2898–2910.
- Miura S, Matsumoto K, Oki A *et al.* Do we need a different strategy for HPV screening and vaccination in East Asia? *Int J Cancer* 2006; **119**: 2713–2715.
- Kocken M, Helmerhorst TJ, Berkhof J *et al.* Risk of recurrent high-grade cervical intraepithelial neoplasia after successful treatment: A long-term multi-cohort study. *Lancet Oncol* 2011; **12**: 441–450.
- Mitchell MF, Tortolero-Luna G, Cook E *et al.* A randomized clinical trial of cryotherapy, laser vaporization, and loop electrosurgical excision for treatment of squamous intraepithelial lesions of the cervix. *Obstet Gynecol* 1998; **92**: 737–744.
- Nuovo J, Melnikow J, Willan AR *et al.* Treatment outcomes for squamous intraepithelial lesions. *Intl J Gynecol Obstet* 2000; **68**: 25–33.
- Soutter WP, Sasieni P, Panoskaltis T. Long-term risk of invasive cervical cancer after treatment of squamous cervical intraepithelial neoplasia. *Int J Cancer* 2006; **118**: 2048–2055.

Human Papillomavirus Genotype Distribution in Cervical Intraepithelial Neoplasia Grade 2/3 and Invasive Cervical Cancer in Japanese Women

Yukari Azuma¹, Rika Kusumoto-Matsuo¹, Fumihiko Takeuchi¹, Asami Uenoyama², Kazunari Kondo², Hajime Tsunoda², Kazunori Nagasaka³, Kei Kawana³, Tohru Morisada⁴, Takashi Iwata⁴, Daisuke Aoki⁴ and Iwao Kukimoto^{1,*}

¹WHO HPV LabNet Regional Reference Laboratory, WHO Western Pacific Region, Pathogen Genomics Center, National Institute of Infectious Diseases, Tokyo, ²Department of Obstetrics and Gynecology, NTT Medical Center Tokyo, Tokyo, ³Department of Obstetrics and Gynecology, Graduate School of Medicine, The University of Tokyo, Tokyo and ⁴Department of Obstetrics and Gynecology, Keio University School of Medicine, Tokyo, Japan

*For reprints and all correspondence: I. Kukimoto, Pathogen Genomics Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-murayama, Tokyo 208-0011, Japan. E-mail: ikuki@nih.go.jp

Received 22 May 2014; accepted 18 July 2014

Objective: Human papillomavirus vaccines are being introduced worldwide and are expected to reduce the incidence of cervical cancer. Here we report a cross-sectional study using a validated human papillomavirus genotyping method to reveal the human papillomavirus prevalence and genotype distribution in Japanese women with cervical intraepithelial neoplasia Grade 2/3 and invasive cervical cancer.

Methods: Cervical exfoliated cells were collected from 647 patients with abnormal cervical histology (cervical intraepithelial neoplasia Grade 2, $n = 164$; cervical intraepithelial neoplasia Grade 3, $n = 334$; and invasive cervical cancer, $n = 149$), and subjected to the PGMY-PCR-based genotyping assay. The association between human papillomavirus infection and lesion severity was calculated using a prevalence ratio.

Results: Overall, the prevalence of human papillomavirus deoxyribonucleic acid was 96.3% in cervical intraepithelial neoplasia Grade 2, 98.8% in cervical intraepithelial neoplasia Grade 3 and 88.0% in invasive cervical cancer (97.8% in squamous cell carcinoma and 71.4% in adenocarcinoma). The three most prevalent types were as follows: human papillomavirus 16 (29.3%), human papillomavirus 52 (27.4%) and human papillomavirus 58 (22.0%) in cervical intraepithelial neoplasia Grade 2; human papillomavirus 16 (44.9%), human papillomavirus 52 (26.0%) and human papillomavirus 58 (17.4%) in cervical intraepithelial neoplasia Grade 3; and human papillomavirus 16 (47.7%), human papillomavirus 18 (23.5%) and human papillomavirus 52 (8.7%) in invasive cervical cancer. The prevalence ratio of human papillomavirus 16 was significantly higher in cervical intraepithelial neoplasia Grade 3 compared with cervical intraepithelial neoplasia Grade 2 (prevalence ratio, 1.62; 95% confidence interval, 1.26–2.13) and in squamous cell carcinoma compared with cervical intraepithelial neoplasia Grade 3 (prevalence ratio, 1.55; 95% confidence interval, 1.25–1.87). Multiple infections decreased from cervical intraepithelial neoplasia Grade 2/3 (38.4/29.6%) to invasive cervical cancer (14.1%), whereas co-infections with human papillomavirus 16/52/58 were found in cervical intraepithelial neoplasia Grade 2/3.

Conclusions: The results of this study provide pre-vaccination era baseline data on human papillomavirus type distribution in Japanese women and serve as a reliable basis for monitoring the future impact of human papillomavirus vaccination in Japan.

Key words: human papillomavirus – genotyping – cervical cancer – prevalence ratio

INTRODUCTION

Persistent infection with a subset of human papillomaviruses (HPVs), known as high-risk HPVs, is a primary cause of the development of cervical precancerous lesions and invasive cervical cancer (ICC) (1). At least 15 genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) are recognized as high-risk HPVs (2), among which HPV16 is most frequently detected in ICC cases worldwide, followed by HPV18. Recent worldwide introduction of HPV vaccines targeting HPV16/18 (Cervarix[®] and Gardasil[®]) is expected to prevent incident HPV16/18 infection, thereby reducing cervical cancer cases (3). However, clinical trials on HPV vaccines have so far only evaluated its efficacy in preventing precancerous lesions, including cervical intraepithelial neoplasia (CIN) Grades 2 and 3, as a surrogate clinical endpoint, and its final effect on reducing ICC cases is not yet proven. Because the progression into ICC generally requires >10 years of persistent HPV infection, HPV type distribution in CIN2/3 lesions in the general population provides an early indicator to assess the effectiveness of HPV vaccination and thus to estimate any subsequent reduction in ICC cases.

East Asian countries including China, Korea and Japan show region-specific variation in HPV type distribution in ICC cases (4). In particular, HPV52 and HPV58 are more prevalent in these countries compared with Europe, North America and Africa (5, 6). Previous studies report that HPV16/18 cause the majority of ICC cases in Japan (7–10), ranging from 50 to 70%, whereas HPV52/58 are individually detected in ~7% of Japanese ICC cases. Since the HPV vaccines against HPV16/18 infection have exhibited only a limited efficacy for cross protection against other high-risk HPVs (11) it is important to monitor the prevalence of HPV52/58 in CIN2/3 and ICC cases in order to evaluate whether type-replacement occurs in post-vaccination era Japan.

In this study, we used a validated HPV genotyping method (12) to record the most recent data on the prevalence and type distribution of high-risk HPVs in Japanese women with CIN2/3 lesions and ICC. The results provide reliable baseline data on the HPV type distribution in Japanese women with precancerous lesions and cervical cancer that will enable accurate assessment of any future impact from HPV vaccination in Japan.

PATIENTS AND METHODS

STUDY SUBJECTS AND SPECIMEN COLLECTION

We enrolled 647 Japanese women who were histologically diagnosed with CIN2/3 or ICC by punch biopsy or cervical conization (CIN2, $n = 164$; CIN3, $n = 334$; and ICC, $n = 149$) at three hospitals in the Tokyo metropolitan area (NTT Medical Center Tokyo, Keio University Hospital, and The University of Tokyo Hospital) from September 2009 to December 2013. Histological diagnosis was made using hematoxylin–eosin-stained sections according to the World Health Organization (WHO) classification by experienced

pathologists at each hospital. When diagnoses between punch biopsy and cervical conization were discordant, a higher grade of histology was taken as final diagnosis. The mean age \pm standard deviation and age range in each histological grade was as follows: CIN2, 36.4 ± 7.7 years (21–62 years); CIN3, 38.9 ± 8.0 years (21–67 years); ICC, 48.0 ± 14.9 years (27–88 years). In Japan, Cervarix[®] and Gardasil[®] were approved for use in 2009 and 2011, respectively, but all the study participants reported no history of HPV vaccination except for one CIN2 case that had recently been administered with Gardasil[®].

Before histopathological diagnosis, cervical exfoliated cells were collected in Thinprep[®] media using a Cervex-brush[®] combi for subsequent HPV genotyping. The study protocol was approved by the Ethics Committee at each hospital and the National Institute of Infectious Diseases, and written informed consent for study participation was obtained from each patient.

HPV GENOTYPING

DNA extraction and HPV genotyping were centralized in a laboratory at the National Institute of Infectious Diseases. Total DNA was extracted from a 200- μ l aliquot of cervical exfoliated cells using the QIAamp DNA Blood Mini Kit (Qiagen) and a MagNA Pure LC 2.0 (Roche Diagnostic). An aliquot of the purified DNA was then used for PCR amplification with AmpliTaq Gold[®] polymerase (GE Healthcare Bio-Sciences), biotinylated PGMY09/11 primers to amplify the L1 DNA of mucosal HPVs, and biotinylated HLA primers to amplify cellular HLA DNA. Positive control (0.1 pg/mL of HPV16 DNA as a plasmid) and negative control (dH₂O) were included to verify the sensitivity of PCR and monitor contamination of HPV DNA in reagents. The PCR products were run on 1.5% agarose gels to assign the positivity of HPV DNA amplification and to confirm the integrity of the extracted DNA by amplification of HLA DNA. Reverse blotting hybridization was performed as described (12, 13). Briefly, 15 μ l denatured PCR products were allowed to hybridize with oligonucleotide probes specific for 31 HPV types (HPV6, 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 68, 69, 70, 73, 82, 83, and 84) immobilized on a Biotodyne C membrane (Pall corporation) using a Miniblotter MN45 (Immunitics, Cambridge, MA, USA). The hybridized DNA was detected using streptavidin–HRP (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and the ECL detection reagent (GE Healthcare Bio-Sciences). For adenocarcinoma samples with negative results from the L1 PCR, E6 PCR was performed using PCR Human Papillomavirus Typing Set (Takara, Ohtsu, Japan) that detects HPV16, 18, 31, 33, 35, 52 and 58.

STATISTICAL ANALYSIS

A generalized linear model with binomial distribution and log link was used to calculate the prevalence ratio (PR) of high-risk HPVs between different histological grades with 95% confidence intervals (CI). The PR was adjusted with the

Table 1. Human papillomavirus (HPV) genotype distribution in CIN2/3 in Japanese women

| Type | CIN2 (n = 164) | % | CIN3 (n = 334) | % | PR (CIN3 vs. CIN2) (95% CI) | P (Wald test) |
|------------------|----------------|------|----------------|------|-----------------------------|-----------------|
| High risk | | | | | | |
| 16 | 48 | 29.3 | 150 | 44.9 | 1.62 (1.26–2.13) | 0.0003** |
| 18 | 11 | 6.7 | 24 | 7.2 | 1.03 (0.53–2.15) | 0.93 |
| 26 | 1 | 0.6 | 0 | 0.0 | ND | |
| 31 | 16 | 9.8 | 42 | 12.6 | 1.30 (0.77–2.32) | 0.35 |
| 33 | 6 | 3.7 | 15 | 4.5 | 1.33 (0.54–3.69) | 0.55 |
| 35 | 5 | 3.0 | 6 | 1.8 | 0.63 (0.19–2.19) | 0.45 |
| 39 | 6 | 3.7 | 8 | 2.4 | 0.67 (0.23–2.03) | 0.46 |
| 45 | 4 | 2.4 | 3 | 0.9 | 0.35 (0.07–1.61) | 0.18 |
| 51 | 16 | 9.8 | 20 | 6.0 | 0.65 (0.34–1.25) | 0.18 |
| 52 | 45 | 27.4 | 87 | 26.0 | 0.93 (0.68–1.28) | 0.63 |
| 53 | 10 | 6.1 | 4 | 1.2 | 0.23 (0.06–0.69) | 0.013* |
| 56 | 9 | 5.5 | 8 | 2.4 | 0.43 (0.16–1.12) | 0.08 |
| 58 | 36 | 22.0 | 58 | 17.4 | 0.81 (0.56–1.20) | 0.28 |
| 59 | 1 | 0.6 | 1 | 0.3 | 0.41 (0.02–10.5) | 0.53 |
| 66 | 3 | 1.8 | 3 | 0.9 | 0.39 (0.08–1.78) | 0.22 |
| 68 | 5 | 3.0 | 5 | 1.5 | 0.66 (0.18–2.35) | 0.51 |
| 73 | 0 | 0.0 | 0 | 0.0 | ND | |
| 82 | 8 | 4.9 | 10 | 3.0 | 0.66 (0.26–1.72) | 0.38 |
| Low risk | | | | | | |
| 6 | 3 | 1.8 | 6 | 1.8 | | |
| 11 | 2 | 1.2 | 0 | 0.0 | | |
| 40 | 1 | 0.6 | 0 | 0.0 | | |
| 42 | 1 | 0.6 | 0 | 0.0 | | |
| 43 | 0 | 0.0 | 0 | 0.0 | | |
| 44 | 1 | 0.6 | 0 | 0.0 | | |
| 54 | 2 | 1.2 | 2 | 0.6 | | |
| 55 | 4 | 2.4 | 2 | 0.6 | | |
| 57 | 0 | 0.0 | 0 | 0.0 | | |
| 69 | 3 | 1.8 | 1 | 0.3 | | |
| 70 | 2 | 1.2 | 1 | 0.3 | | |
| 83 | 0 | 0.0 | 1 | 0.3 | | |
| 84 | 0 | 0.0 | 2 | 0.6 | | |
| Negative | 6 | 3.7 | 4 | 1.2 | | |
| Multiple | 63 | 38.4 | 99 | 29.6 | | |

Single and multiple infections combined.
 ** $P < 0.001$; * $P < 0.05$; ND, not determined. Statistically significant values are indicated in boldface.
 One case having HPV vaccination history (HPV16, 18, 53 and 58 positive) is included in CIN2.
 CIN2, cervical intraepithelial neoplasia Grade 2; CIN3, cervical intraepithelial neoplasia Grade 3; PR, prevalence ratio; CI, confidence interval.

women’s age at the time of diagnosis. Pearson’s χ^2 test with Yates’ continuity correction was used to examine differences in the proportion of HPV infections. Two-sided P values were calculated and considered to be significant at <0.05 . All statistical analyses were performed using R version 2.11.1.

RESULTS

HPV PREVALENCE AND TYPE DISTRIBUTION

Overall, HPV DNA was detected in 158 of the 164 CIN2 cases (96.3%), 330 of the 334 CIN3 cases (98.8%) and 131 of

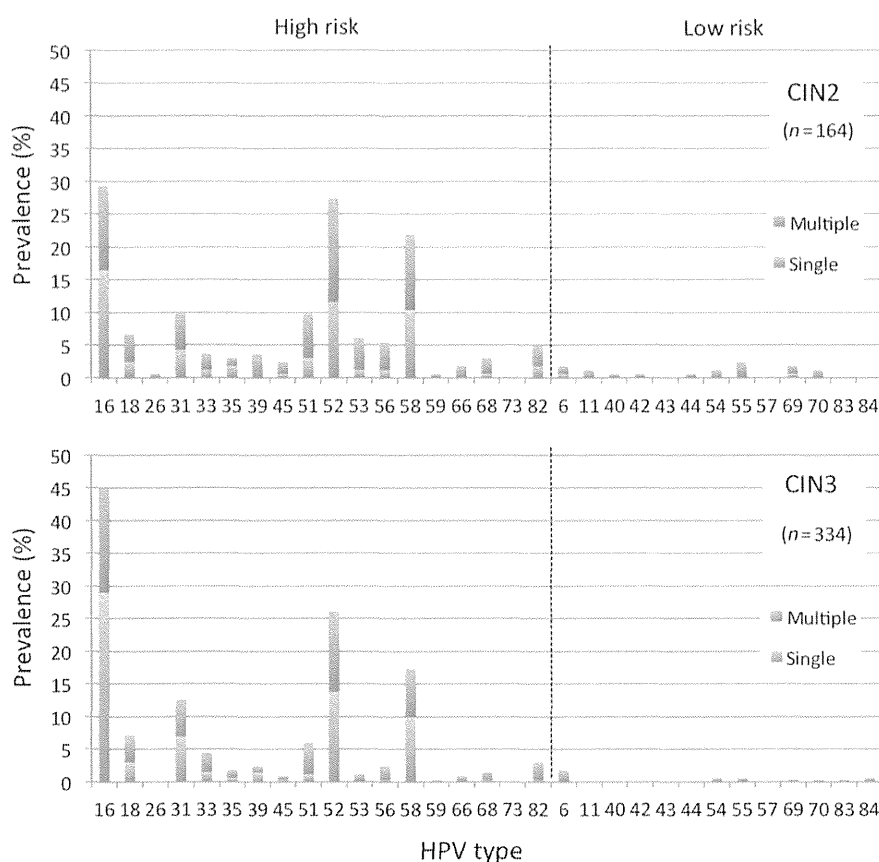


Figure 1. Human papillomavirus (HPV) type distribution in cervical intraepithelial neoplasia (CIN) Grade 2/3 in Japanese women. The proportions of single and multiple infections are presented for individual types. HPV types are grouped as either high- or low-risk based on the risk classification of Munoz et al. (2), except that HPV26 and HPV53 are included in the high-risk group based on phylogenetic classification of the L1 nucleotide sequences.

the 149 ICC cases (87.9%). Table 1 and Figure 1 show the prevalence and genotype distribution of both high- and low-risk HPV types in the CIN2/3 cases that were enrolled in this study. In CIN2 and CIN3, three genotypes, HPV16, HPV52 and HPV58, were predominantly detected among 31 HPV types examined. The proportion of these HPV types in each histological stage was as follows: HPV16 (29.3%), HPV52 (27.4%) and HPV58 (22.0%) in CIN2; HPV16 (44.9%), HPV52 (26.0%) and HPV58 (17.4%) in CIN3. Low-risk HPV types were detected in 18 cases (11.0%) in CIN2 and 14 cases (4.2%) in CIN3, all with other high-risk HPV types except for one case of single-positive HPV6 in CIN2 and one case of single-positive HPV69 in CIN2. The proportion of low-risk HPV infections was significantly lower in CIN3 than in CIN2 ($P = 0.007$, χ^2 test).

In accordance with the distribution patterns observed with CIN2/3 lesions, HPV16 (47.7%) was most frequently detected in ICC, whereas the second common type was HPV18 (23.5%), followed by HPV52 (8.7%) (Table 2). Among the ICC cases, the detection rate of HPV DNA was relatively low in adenocarcinoma (71.4%) compared with squamous cell carcinoma (SCC) (97.8%), and type distributions of high-risk

HPV types were apparently different between the two histological types (Fig. 2), as previously reported (14). Although HPV16 (60.4%) was the most common type in SCC, followed by HPV52 (12.1%) and HPV18 (11.0%), HPV18 (41.1%) was most frequently detected in adenocarcinoma, followed by HPV16 (28.6%) and HPV51 (5.4%).

PREVALENCE RATIO

To evaluate the risk of CIN progression attributable to individual high-risk HPV types, the prevalence ratio (PR) of each high-risk HPV was calculated by comparing the incidence in CIN3 and CIN2. As shown in Table 1, HPV16 prevalence was significantly higher in CIN3 compared with CIN2 (PR = 1.62, 95% CI = 1.26–2.13). Conversely, HPV53 prevalence was negatively associated with progression from CIN2 to CIN3 (PR = 0.23, 95% CI = 0.06–0.69). CIN2/3 and SCC cases positive for HPV16 showed a trend towards younger age compared with HPV16-negative cases (Fig. 3). Indeed, statistical analysis, using a log-binomial model, of high-risk HPV prevalence in CIN2 and CIN3 with age revealed significantly higher PR with decreasing age for HPV16 (PR/year = 1.03,

Table 2. HPV genotype distribution in ICC in Japanese women

| Type | ICC (n = 149) | % | SCC (n = 91) | % | Adc (n = 56) | % | PR (SCC vs. CIN3) (95% CI) | P (Wald test) |
|------------------|---------------|------|--------------|------|--------------|------|----------------------------|------------------|
| High risk | | | | | | | | |
| 16 | 71 | 47.7 | 55 | 60.4 | 16 | 28.6 | 1.55 (1.25–1.87) | 0.00007** |
| 18 | 35 | 23.5 | 10 | 11.0 | 23 | 41.1 | 1.62 (0.72–3.33) | 0.20 |
| 26 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | ND | |
| 31 | 3 | 2.0 | 3 | 3.3 | 0 | 0.0 | 0.23 (0.05–0.65) | 0.017* |
| 33 | 4 | 2.7 | 3 | 3.3 | 1 | 1.8 | 0.70 (0.15–2.22) | 0.59 |
| 35 | 2 | 1.3 | 2 | 2.2 | 0 | 0.0 | 1.69 (0.24–7.38) | 0.52 |
| 39 | 3 | 2.0 | 3 | 3.3 | 0 | 0.0 | 1.07 (0.20–4.14) | 0.93 |
| 45 | 1 | 0.7 | 1 | 1.1 | 0 | 0.0 | 0.98 (0.04–9.24) | 0.99 |
| 51 | 3 | 2.0 | 0 | 0.0 | 3 | 5.4 | ND | |
| 52 | 13 | 8.7 | 11 | 12.1 | 2 | 3.6 | 0.47 (0.24–0.82) | 0.014* |
| 53 | 1 | 0.7 | 0 | 0.0 | 0 | 0.0 | ND | |
| 56 | 3 | 2.0 | 2 | 2.2 | 1 | 1.8 | 0.93 (0.13–3.97) | 0.93 |
| 58 | 8 | 5.4 | 6 | 6.6 | 2 | 3.6 | 0.38 (0.15–0.80) | 0.024* |
| 59 | 1 | 0.7 | 0 | 0.0 | 1 | 1.8 | ND | |
| 66 | 2 | 1.3 | 1 | 1.1 | 1 | 1.8 | 0.57 (0.02–6.46) | 0.69 |
| 68 | 3 | 2.0 | 3 | 3.3 | 0 | 0.0 | 3.26 (0.67–13.0) | 0.11 |
| 73 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | ND | |
| 82 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | ND | |
| Low risk | | | | | | | | |
| 42 | 1 | 0.7 | 0 | 0.0 | 1 | 1.8 | | |
| 54 | 3 | 2.0 | 3 | 3.3 | 0 | 0.0 | | |
| Negative | 18 | 12.1 | 2 | 2.2 | 16 | 28.6 | | |
| Multiple | 21 | 14.1 | 13 | 14.3 | 7 | 12.5 | | |

Single and multiple infections combined.

** $P < 0.001$; * $P < 0.05$; ND, not determined. Statistically significant values are indicated in boldface.

One adenocarcinoma (HPV18 positive) is included in Adc. One small cell carcinoma (HPV18 positive) and one undifferentiated carcinoma (HPV18/53 positive) included in SCC are excluded from SCC and Adc.

ICC, invasive cervical cancer; SCC, squamous cell carcinoma; Adc, adenocarcinoma; CIN3, cervical intraepithelial neoplasia Grade 3; PR, prevalence ratio; CI, confidence interval.

95% CI = 1.01–1.04, $P = 0.0009$) and for HPV68 (PR/year = 1.13, 95% CI = 1.03–1.27, $P = 0.021$). No significant association with age was observed for prevalence of other high-risk types (data not shown).

When the prevalence of high-risk types was analyzed between CIN3 and SCC, an excess of HPV16 was found in SCC compared with CIN3 (HPV16: PR = 1.55, 95% CI = 1.25–1.87) (Table 2). In contrast, the prevalence of HPV31, HPV52 and HPV58 was significantly decreased in SCC compared with CIN3.

MULTIPLE INFECTIONS

As shown in Figure 4A, multiple infections were detected in 38.4% of the CIN2 cases, 29.6% of the CIN3 cases and 14.1% of the ICC cases, showing a decreasing trend with severity of

lesions, whereas the proportion of single infection increased from 57.9% in CIN2 to 73.8% in ICC. The number of detected HPV types significantly decreases as the lesion develops to ICC ($P = 0.0005$) and with age ($P = 1.3 \times 10^{-6}$). Among multiple infections, the proportion of HPV16 and/or HPV18 infections with other high-risk HPVs was 15.9% in CIN3, which was significantly higher than the 6.7% in ICC ($P = 0.009$), but not significantly different to the 12.2% in CIN2 ($P = 0.34$) (Fig. 4B). The proportion of HPV16 and/or HPV18 infections without other high-risk HPVs was significantly higher in CIN3 than in CIN2 ($P = 0.006$), and was higher still in ICC than in CIN3 ($P = 1.7 \times 10^{-8}$). In contrast, the proportion of high-risk HPV infections other than HPV16/18 was significantly lower in CIN3 than in CIN2 ($P = 0.010$), and was yet lower in ICC than in CIN3 ($P = 1.8 \times 10^{-9}$).

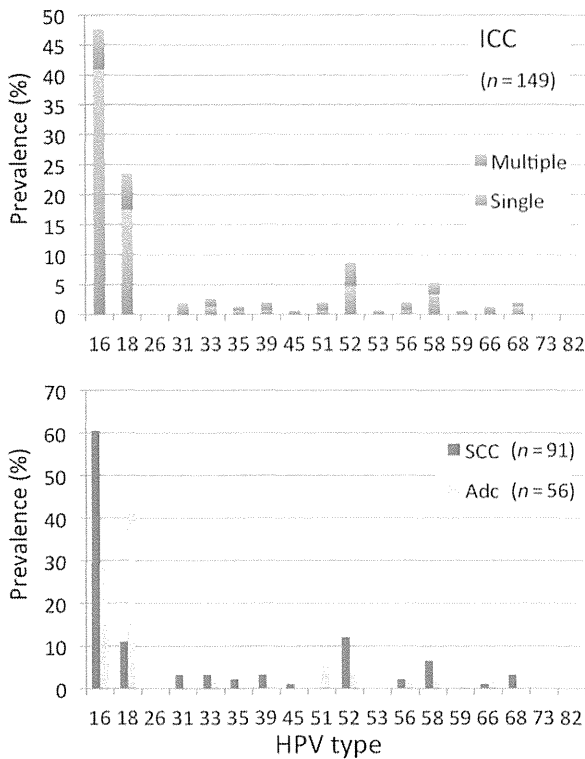


Figure 2. HPV type distribution in invasive cervical cancer (ICC) in Japanese women. The prevalence of single and multiple infections in ICC for individual types (upper panel). The type distribution in squamous cell carcinoma (SCC) and adenocarcinoma (Adc) (lower panel).

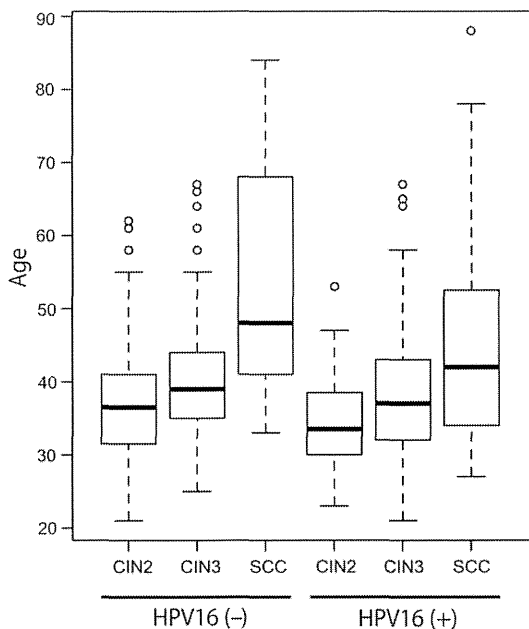


Figure 3. Association of age with HPV16 positivity in CIN2/3 and SCC.

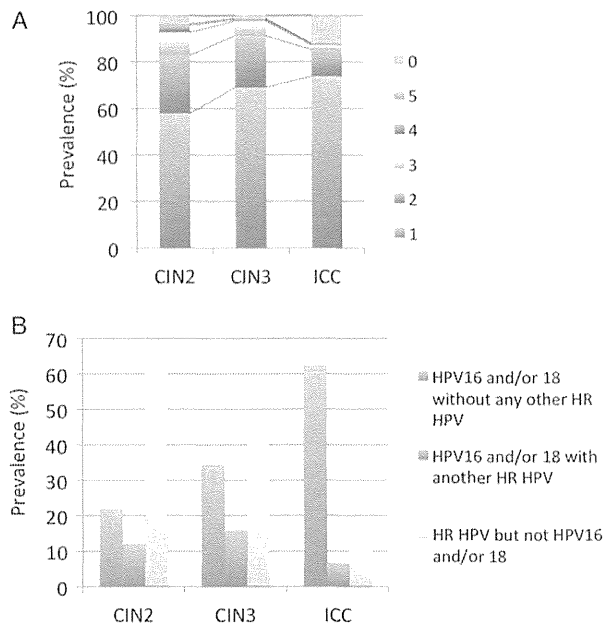


Figure 4. Multiple HPV infections in CIN2/3 and ICC in Japanese women. (A) The distribution of the number of HPV types detected for each histological grade. (B) The prevalence of HPV16 and/or 18 and other high-risk HPVs, alone or in mixed infections with HPV16 and/or 18, for each histological grade.

With regard to the three most frequent types detected in CIN2/3, the proportion of co-infections was as follows: in CIN2, HPV16/52 in 8 cases (4.9%), HPV16/58 in 4 cases (2.4%), and HPV52/58 in 8 cases (4.9%); in CIN3, HPV16/52 in 14 cases (4.2%), HPV16/58 in 9 cases (2.7%), and HPV52/58 in 8 cases (2.4%) (Fig. 5). Triple-infections were found in two cases of CIN2 and in one case of CIN3.

DISCUSSION

Here we have presented the prevalence and type distribution of high-risk HPVs in cervical precancerous lesions and ICC in Japan using a validated genotyping method. A number of studies have so far been conducted to investigate the HPV type distribution in Japanese women (8–10, 15–19), but most of those studies depended on HPV typing performed at each hospital laboratory and only a limited number utilized a centralized external laboratory with quality assurance of its testing capability. Since our typing capability using the PGMY-lineblot assay has been consistently evaluated as proficient in the HPV DNA proficiency panel studies conducted annually by the WHO HPV laboratory network (20), the results obtained in this study provide the most recent and reliable pre-vaccination baseline data for Japanese HPV infection.

In a meta-analysis assembling HPV genotyping data from 984 ICC cases in Japan, the top three HPV types were HPV16 (44.8%), HPV18 (14.0%), and HPV52 (7.0%) (7). The

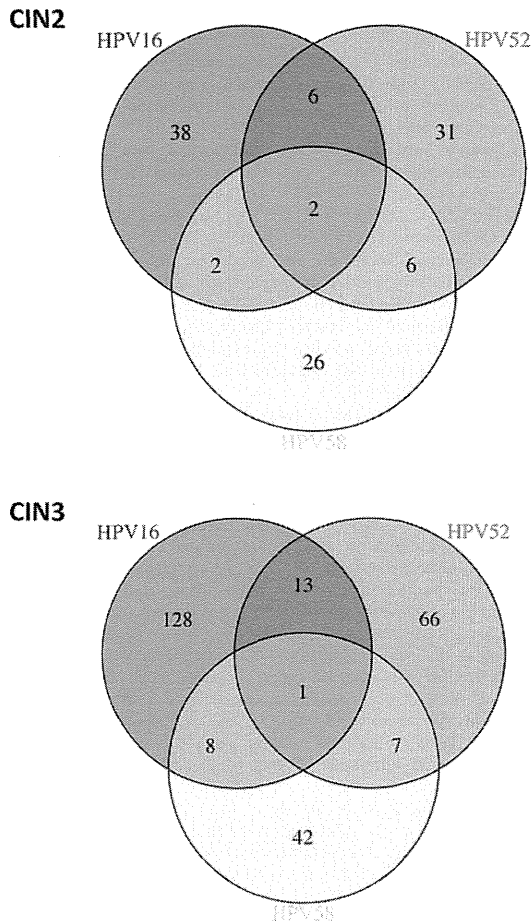


Figure 5. Venn diagram showing the overlap of HPV16/52/58 infections in CIN2 (A) and CIN3 (B). The number in circles indicates the number of subjects positive for HPV16 and/or HPV52 and/or HPV58.

observed distribution of high-risk HPVs in ICC reported in this study is almost similar, suggesting that the general trend for causative HPV types in ICC has not dramatically changed in Japan. The type distributions in SCC and adenocarcinoma found in this study are also similar to those reported in the previous meta-analysis (7), in which the top three most frequent HPV types were HPV16 (45.8%), HPV18 (10.8%) and HPV52 (7.4%) in SCC, and HPV18 (58.2%), HPV16 (31.3%) and HPV68 (4.5%) in adenocarcinoma. However, HPV68 was not detected in adenocarcinoma in this study.

Compared with the high HPV positivity in SCC (97.8%), the low HPV detection rate observed in adenocarcinoma (71.4%) is consistent with a recent study summarizing HPV genotyping data for cervical adenocarcinoma (21), which demonstrated that the positivity rate of HPV DNA in adenocarcinoma ranged from 65.6% (6) to 82.0% (22). The HPV-negative adenocarcinoma cases ($n = 16$) in this study were further examined by PCR targeting the HPV E6 gene, but again no HPV DNA was amplified from these samples

(data not shown). The low detection rate of HPV DNA may be due to HPV integration into the host genome that disrupts the L1 and E6 genes used for HPV typing, improper sampling of endocervical cells, or degradation of cell samples containing low levels of HPV DNA. The low HPV positivity in cervical adenocarcinoma may also be attributed to the presence of inherently HPV-unrelated glandular lesions. In support of this notion, gastric-type adenocarcinoma, which exhibits a range of phenotypic gastric differentiation, has recently been proposed as another subtype of cervical adenocarcinoma and shown to be unrelated to HPV infection (23). Nevertheless, gastric-type adenocarcinoma was not found in the adenocarcinoma cases in this study.

The HPV type distribution in CIN2/3 in this study shows similar patterns to that previously reported by Onuki et al. (17), in which the top three most frequent types were HPV16 (24.1%), HPV52 (17.5%) and HPV58 (10.7%). Thus, the results in this study strongly support a major role for HPV16, HPV52 and HPV58 in causing CIN2/3 in Japanese women.

A recent prospective study followed Japanese women with low-grade cervical lesions and estimated the risk of disease progression associated with high-risk HPV infections (24). That study reported hazard ratios of individual high-risk types for progression to CIN3; 7 HPV types (HPV16, 18, 31, 33, 35, 52 and 58) showed a high risk of progression. Consistent with these findings, in our study HPV16 exhibited significantly higher PR in CIN3 compared with CIN2, suggesting a higher potential for progression from CIN2 to CIN3 than with other high-risk types. Faster progression of HPV16-infected lesions to CIN3 can also explain the observed association of younger age with the development of HPV16-positive CIN3. The high PR of HPV16 in SCC compared with CIN3 lends further support to the increased carcinogenicity of persistent HPV16 infection. In contrast, we found a low prevalence of HPV31, HPV52 and HPV58 in SCC compared with CIN3, which suggests a lower potential for progression to SCC than with HPV16 infection.

Multiple infections were more frequently detected in CIN2/3 in our results than in those reported by Onuki et al. (11.3% in CIN2/3) (17). This difference likely reflects the higher sensitivity of the PGMV-line blot genotyping methodology to detect multiple infections, without inter-type PCR competition, as previously reported (25). Alternatively, the high prevalence of multiple infections might result from using cervical exfoliated cell instead of tissue sections containing CIN2/3 lesions. However, multiple infections with high-risk types have previously been reported in a study using tissue sections from cervical biopsies (26).

Caution should be taken regarding co-infections of HPV16/18 with other high-risk types, because currently available vaccines targeting HPV16/18 exhibit only limited cross protection against infections with other high-risk HPVs (3). We report a substantial proportion of HPV16/18 co-infections with other high-risk HPVs in CIN3 (Fig. 4B), a proportion that is significantly higher than that in ICC. Although a causative HPV type for CIN3 lesions is difficult to determine when

co-infections are detected, the co-infection may still progress to ICC via the other high-risk types if HPV16/18 infections were prevented by vaccination. Of particular concern is the fact that HPV52 and HPV58 are commonly detected in East Asian countries and we report a significant number of co-infections between HPV52/58 and HPV16 in CIN2/3 in Japanese women (Fig. 5). Therefore, there is a possibility of an increase in the incidence of HPV52/58-positive ICC if HPV16/18 infections are prevented. Thus, careful monitoring of these genotypes in CIN2/3 lesions will be required after the widespread introduction of HPV vaccines into Japan and other East Asian countries in order to evaluate the overall efficacy of HPV vaccination.

Acknowledgement

We thank Dr Tadahito Kanda for critical reading of the manuscript.

Funding

This study was supported by funding from the Ministry of Health, Labor, and Welfare in Japan.

Conflict of interest statement

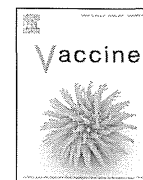
None declared.

References

1. The FUTUREII Study Group. Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions. *N Engl J Med* 2007;356:1915–27.
2. Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003;348:518–27.
3. Schiller JT, Castellsague X, Garland SM. A review of clinical trials of human papillomavirus prophylactic vaccines. *Vaccine* 2012; 30(Suppl 5):F123–138.
4. Parkin DM, Louie KS, Clifford G. Burden and trends of type-specific human papillomavirus infections and related diseases in the Asia Pacific region. *Vaccine* 2008; 26(Suppl 12):M1–16.
5. Konno R, Shin HR, Kim YT, Song YS, Sasagawa T, et al. Human papillomavirus infection and cervical cancer prevention in Japan and Korea. *Vaccine* 2008; 26(Suppl 12):M30–42.
6. de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE, et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Lancet Oncol* 2010;11:1048–56.
7. Miura S, Matsumoto K, Oki A, Satoh T, Tsunoda H, et al. Do we need a different strategy for HPV screening and vaccination in East Asia? *Int J Cancer* 2006;119:2713–5.
8. Yoshikawa H, Kawana T, Kitagawa K, Mizuno M, Yoshikura H, et al. Detection and typing of multiple genital human papillomaviruses

- by DNA amplification with consensus primers. *Jpn J Cancer Res* 1991;82:524–31.
9. Asato T, Maehama T, Nagai Y, Kanazawa K, Uezato H, et al. A large case–control study of cervical cancer risk associated with human papillomavirus infection in Japan, by nucleotide sequencing-based genotyping. *J Infect Dis* 2004;189:1829–32.
10. Nakagawa S, Yoshikawa H, Onda T, Kawana T, Iwamoto A, et al. Type of human papillomavirus is related to clinical features of cervical carcinoma. *Cancer* 1996;78:1935–41.
11. Wheeler CM, Castellsague X, Garland SM, Szarewski A, Paavonen J, et al. Cross-protective efficacy of HPV-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by non-vaccine oncogenic HPV types: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial. *Lancet Oncol* 2012;13:100–10.
12. Estrade C, Menoud PA, Nardelli-Haeffliger D, Sahli R. Validation of a low-cost human papillomavirus genotyping assay based on PGM1 PCR and reverse blotting hybridization with reusable membranes. *J Clin Microbiol* 2011;49:3474–81.
13. World Health Organization. *Human Papillomavirus Laboratory Manual*. 1st edn. (WNO/IVB/10.12) Geneva, Switzerland: WHO 2009. http://whqlibdoc.who.int/hq/2010/WHO_IVB_10.12_eng.pdf (15 August 2014, date last accessed).
14. Bulk S, Berkhof J, Bulkman NW, Zielinski GD, Rozendaal L, et al. Preferential risk of HPV16 for squamous cell carcinoma and of HPV18 for adenocarcinoma of the cervix compared to women with normal cytology in The Netherlands. *Br J Cancer* 2006;94:171–5.
15. Konno R, Tamura S, Dobbelaere K, Yoshikawa H. Prevalence and type distribution of human papillomavirus in healthy Japanese women aged 20 to 25 years old enrolled in a clinical study. *Cancer Sci* 2011;102:877–82.
16. Maehama T, Asato T, Kanazawa K. Prevalence of HPV infection in cervical cytology-normal women in Okinawa, Japan, as determined by a polymerase chain reaction. *Int J Gynaecol Obstet* 2000;69:175–6.
17. Onuki M, Matsumoto K, Satoh T, Oki A, Okada S, et al. Human papillomavirus infections among Japanese women: age-related prevalence and type-specific risk for cervical cancer. *Cancer Sci* 2009;100:1312–6.
18. Sasagawa T, Basha W, Yamazaki H, Inoue M. High-risk and multiple human papillomavirus infections associated with cervical abnormalities in Japanese women. *Cancer Epidemiol Biomarkers Prev* 2001;10:45–52.
19. Takehara K, Toda T, Nishimura T, Sakane J, Kawakami Y, et al. Human papillomavirus types 52 and 58 are prevalent in uterine cervical squamous lesions from Japanese women. *Pathol Res Int* 2011;2011:246936.
20. Eklund C, Zhou T, Dillner J. Global proficiency study of human papillomavirus genotyping. *J Clin Microbiol* 2010;48:4147–55.
21. Pimenta JM, Galindo C, Jenkins D, Taylor SM. Estimate of the global burden of cervical adenocarcinoma and potential impact of prophylactic human papillomavirus vaccination. *BMC Cancer* 2013;13:553.
22. Li N, Franceschi S, Howell-Jones R, Snijders PJ, Clifford GM. Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: Variation by geographical region, histological type and year of publication. *Int J Cancer* 2011;128:927–35.
23. Mikami Y, McCluggage WG. Endocervical glandular lesions exhibiting gastric differentiation: an emerging spectrum of benign, premalignant, and malignant lesions. *Adv Anat Pathol* 2013;20:227–37.
24. Matsumoto K, Oki A, Furuta R, Maeda H, Yasugi T, et al. Predicting the progression of cervical precursor lesions by human papillomavirus genotyping: a prospective cohort study. *Int J Cancer* 2011;128:2898–910.
25. Mori S, Nakao S, Kukimoto I, Kusumoto-Matsuo R, Kondo K, et al. Biased amplification of human papillomavirus DNA in specimens containing multiple human papillomavirus types by PCR with consensus primers. *Cancer Sci* 2011;102:1223–7.
26. Howell-Jones R, Bailey A, Beddows S, Sargent A, de Silva N, et al. Multi-site study of HPV type-specific prevalence in women with cervical cancer, intraepithelial neoplasia and normal cytology, in England. *Br J Cancer* 2010;103:209–16.

Downloaded from <http://jco.oxfordjournals.org/> at University of Tokyo Library on February 11, 2015



Oral vaccination against HPV E7 for treatment of cervical intraepithelial neoplasia grade 3 (CIN3) elicits E7-specific mucosal immunity in the cervix of CIN3 patients



Kei Kawana^{a,*}, Katsuyuki Adachi^a, Satoko Kojima^a, Ayumi Taguchi^a, Kensuke Tomio^a, Aki Yamashita^a, Haruka Nishida^a, Kazunori Nagasaka^a, Takahide Arimoto^a, Terufumi Yokoyama^{b,1}, Osamu Wada-Hiraike^a, Katsutoshi Oda^a, Tomomitsu Sewaki^{b,1}, Yutaka Osuga^a, Tomoyuki Fujii^a

^a Department of Obstetrics and Gynecology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

^b GENOLAC BL Corp., 503, 1-9-26-401 Minamisenba, Chuo-ku, Osaka-shi, Osaka 542-0081, Japan

ARTICLE INFO

Article history:

Received 12 May 2014

Received in revised form 28 July 2014

Accepted 8 September 2014

Available online 22 September 2014

Keywords:

HPV therapeutic vaccine

Mucosal immunity

Cervical intraepithelial neoplasia (CIN) 3

E7-expressing *Lactobacillus*

Oral administration

ABSTRACT

Background: Cervical intraepithelial neoplasia grade 3 (CIN3) is a mucosal precancerous lesion caused by high-risk human papillomavirus (HPV). Induction of immunological clearance of CIN3 by targeting HPV antigens is a promising strategy for CIN3 therapy. No successful HPV therapeutic vaccine has been developed.

Methods: We evaluated the safety and clinical efficacy of an attenuated *Lactobacillus casei* expressing modified full-length HPV16 E7 protein in patients with HPV16-associated CIN3. Ten patients were vaccinated orally during dose optimization studies (1, 2, 4, or 6 capsules/day) at weeks 1, 2, 4, and 8 (Step 1). Seven additional participants were only tested using the optimized vaccine formulation (Step 2), giving a total of 10 patients who received optimized vaccination. Cervical lymphocytes (CxLs) and peripheral blood mononuclear cells (PBMCs) were collected and E7 specific interferon- γ -producing cells were counted (E7 cell-mediated immune responses; E7-CMI) by ELISPOT assay. All patients were re-evaluated 9 weeks after initial vaccine exposure using cytology and biopsy to assess pathological efficacy.

Results: No patient experienced an adverse event. E7-CMI in both CxLs and PBMCs was negligible at baseline. All patients using 4–6 capsules/day showed increased E7-CMI in CxLs, whereas patients using 1–2 capsules/day did not. No patient demonstrated an increase in E7-CMI in their PBMCs. In comparison between patients of cohorts, E7-CMI at week 9 (9 wk) in patients on 4 capsules/day was significantly higher than those in patients on 1, 2, or 6 capsules/day. Most patients (70%) taking the optimized dose experienced a pathological down-grade to CIN2 at week 9 of treatment. E7-CMI in CxLs correlated directly with the pathological down-grade.

Conclusions: Oral administration of an E7-expressing *Lactobacillus*-based vaccine can elicit E7-specific mucosal immunity in the uterine cervical lesions. We are the first to report a correlation between mucosal E7-CMI in the cervix and clinical response after immunotherapy in human mucosal neoplasia.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Human papillomavirus (HPV) is a major risk factor for the development of cervical cancer, the second most common cancer among women [1]. Some 99% of cervical cancer cases are associated with genital infection with oncogenic HPVs. Among them, HPV type 16 (HPV16) infection is most commonly associated with cervical cancer [2–5]. Recent prophylactic HPV vaccines have been shown to prevent genital infection with HPV types 16 and 18 (HPV16/18) and reduce the incidence of HPV16/18-related high-grade CIN [6–10]. However, little effect will likely be noted among

Abbreviations: CIN3, cervical intraepithelial neoplasia grade 3; HPV, human papillomavirus; CxLs, cervical lymphocytes; PBMCs, peripheral blood mononuclear cells; E7-CMI, E7 cell-mediated immune responses; CTL, cytotoxic T lymphocytes.

* Corresponding author. Tel.: +81 3 3815 5411; fax: +81 3 3816 2017.

E-mail address: kkawana-tyk@umin.org (K. Kawana).

¹ Tel.: +81 98 858 8607; fax: +81 98 858 8607.

<http://dx.doi.org/10.1016/j.vaccine.2014.09.020>
0264-410X/© 2014 Elsevier Ltd. All rights reserved.

patients who were already infected by HPV prior to vaccination. The limitations of prophylactic HPV vaccines demonstrate a pressing need for novel approaches, possibly immune-mediated, to eradicate HPV-associated neoplasia and suggest that the development of therapeutic HPV vaccines for the treatment of HPV-associated lesions should remain an important goal [11]. The combined actions of the high-risk HPV E6 and E7 oncoproteins are essential for the maintenance of the neoplastic phenotype. Since E6 and E7 are the only HPV proteins expressed in precursor lesions, they represent reliable antigenic targets for immunotherapy of CIN3. Immunization with E6 and/or E7 of HPV16, with the resultant generation of antigen-specific CTL (cytotoxic T lymphocytes), has been a frequent immunotherapeutic approach for HPV-associated neoplasia and has utilized a wide array of potential vaccine delivery systems [12–23]. Previous clinical trials of HPV therapeutic vaccines have been able to elicit systemic E7-specific type 1 cell-mediated immune responses (systemic E7-CMI) using subcutaneous or intramuscular delivery, but few have studied mucosal E7-specific type 1 cell-mediated immune responses (mucosal E7-CMI). Because CIN lesions develop in the cervical mucosa, local mucosal lymphocytes possessing E7-CMI in the cervix are likely to play a direct role in immunological clearance of CIN lesions.

The lymphocytes involved in mucosal immunity are found in the inductive sites of organized mucosa-associated lymphoid tissues and in a variety of effector sites such as the mucosa of the intestine, respiratory tract, and genital tract. Integrin $\beta 7$ is the most common homing receptor expressed on gut-derived mucosal lymphocytes [24]. We have demonstrated that cervical brushing methodology enables us to preferentially collect integrin $\beta 7^+$ mucosal cervical lymphocytes (CxLs) from CIN lesions [25]. Using murine models, several studies on immunization with *Lactobacillus*-based vaccines have demonstrated an induction of systemic E7-CMI and regression of subcutaneous HPV16 E7-positive tumors [26–28]. However, they have neither provided an insight into mucosal T cell responses to oral vaccination nor into the anti-tumor effects on mucosal intraepithelial neoplasms. We have observed an induction of mucosal E7-CMI within intestinal mucosa after oral administration of *Lactobacillus casei* expressing HPV16 E7 in mice [29]. These studies suggested that oral vaccination may surmount some of the deficiencies seen with systemic immunization in previous CIN therapeutic vaccine clinical trials and encouraged us to embark on a clinical trial using GLBL101c. To assess the safety, immunogenicity, and clinical efficacy of GLBL101c, we designed a Phase I/IIa study involving patients with HPV16-positive CIN3. This is the first clinical trial to use oral vaccination for the treatment of HPV-associated neoplasia.

2. Materials and methods

2.1. Patients

Enrolled patients had (1) histologically confirmed ectocervical CIN3 lesions and were (2) infected only with HPV16 (exclusion of other high-risk HPV types) as documented by in-house PGMY-CHU HPV genotyping methods which can detect 34 HPV subtypes [30]. Other eligibility criteria included: (3) age 18–45, (4) colposcopic evidence of a persistent high-grade lesion 4 weeks after biopsy, (5) normal pretreatment laboratory blood values, and (6) signed informed consent. Exclusion criteria included: (1) any signs of invasive disease, (2) endocervical involvement, (3) pregnancy/lactation, and (4) HIV positivity, immunosuppressive disease or use of immunosuppressive medications.

2.2. Study design

Since the spontaneous regression rate of CIN3 at a 9-week time point is thought to be less than 10% [13,31,32], this study was designed as a single-center, single-arm (non-controlled), observational Phase I/IIa study. The primary end points were to evaluate the safety and the pathological efficacy of vaccination and the secondary endpoints were mucosal and systemic HPV16 E7-CMI and local cytological efficacy. For initial safety assessments, the minimal formulated amount (250 mg) of GLBL101c (one capsule) was administered daily. Next, four small scale dose-escalation cohorts (one or three patients per cohort) were treated with 1, 2, 4, or 6 capsules/day for four total rounds (Step 1). If no adverse effects were observed in cohorts given lower doses, the dose was escalated by one capsule per day in the next cohort. If no clinical response was observed at a given dose, the trial was discontinued for that dose level. Once a safe and effective dose was identified, seven more patients were enrolled at that dose level for a total of ten patients using the optimized dose of GLBL101c (Step 2). All patients received four rounds of oral vaccination at week 1, 2, 4, and 8. Each dose of GLBL101c was administered orally once each morning after fasting for five days, each treatment week. We followed our subjects for only 9 weeks prior to reassessment and possible treatment to ensure optimal patient safety. All vaccinations were performed from February 2009 to November 2012. The study was sponsored by the Ministry of Health, Labour and Welfare of Japan for the Third-Term Comprehensive Strategy for Cancer Control, and for Comprehensive Strategy for Practical Medical Technology, Japan, and approved by the medical ethics committee of the University of Tokyo, Faculty of Medicine. All patients gave written informed consent. All data underwent independent third-party management and analysis and were evaluated by a third-party committee for efficacy and safety.

2.3. Composition of the vaccine

GLBL101c was provided by GENOLAC BL Corp (Japan), generated from a recombinant *L. casei* expressing mutated HPV16 E7 as previously described and attenuated using heat. Briefly, the HPV16 E7 gene was modified by inserting point mutations into the Rb-binding site (the D, C, and E in E7 aa21, aa24, and aa26, respectively, were all replaced by a G) [29]. Through these mutations, the carcinogenicity of E7 was abrogated, but its immunogenicity remained intact [33]. The attenuated *L. casei* were purified by washing several times with distilled water then dried to powder. 250 mg of GLBL101c powder was enclosed in a capsule designed to degrade in the bowel.

2.4. Collection and processing of cervical specimens

Cervical cells were collected using a Digene cytobrush as described previously [34]. The cytobrush was inserted into the cervical os and rotated several times. The cytobrush was then placed into a 15-mL tube containing R10 media (RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS), 100 mg/mL streptomycin, and 2.5 μ g/mL amphotericin B) and an anticoagulant (0.1 IU/mL of heparin and 8 nM EDTA). After incubating the sample with 5 mM DL-dithiothreitol at 37 °C for 15 min with shaking, the cytobrush was removed. The tube was centrifuged at 330 \times g for 4 min. The pellet was resuspended in 10 mL of 40% Percoll, layered onto 70% Percoll, and centrifuged at 480 \times g for 18 min. The mononuclear cells at the Percoll interface were removed and washed with PBS. Cell viability was >95%, as confirmed by trypan blue exclusion, and it is noted that all samples were frozen until use for further immunological assay [25].

2.5. Assessment of clinical efficacy

Visual examinations and histological and cytological specimen collections were performed under colposcopy. Biopsies of the same site were obtained at baseline and one week after the final vaccination round (at week 9). As a primary end point, grading of the lesions was performed by several experienced independent blinded pathologists according to strict criteria [35]. If CIN3 was downgraded to CIN2 or less, further surgical intervention was averted. Otherwise, patients with CIN3 underwent cervical conization or laser ablation. We used pathological response criteria that were modified based on the previous study [13]. Patients who did not receive surgical treatment had repeat cytologic evaluation at 6 and 12 months.

2.6. Safety and tolerability

Clinical assessments, laboratory testing and adverse events monitoring were conducted after each round of vaccination. Adverse events were graded according to version 3.0 of the Common Terminology Criteria for Adverse Events (CTCAE), which grades events on a scale of 1–5, with higher grades indicating greater severity.

2.7. Immunological responses to HPV16 E7 (E7-CMI)

All lymphocyte samples were frozen immediately after isolation and stocked in -40°C freezer until use for immunological assay. Approximately 1×10^6 cervical lymphocytes were isolated from each patient's cervix. 5×10^4 cervical lymphocytes (CxLs) or peripheral blood mononuclear cells (PBMCs) were incubated for 24 h at 37°C with antigen presenting cells or 5×10^4 PBMCs were treated with mitomycin C ($75 \mu\text{g}/\text{mL}$, Nakarai, Japan) and washed four times with PBS [25]. Ten microliters of synthesized peptides (working concentration $1 \mu\text{g}/\text{mL}$) covering the entire 98 aa HPV16 E7 amino acid sequence with 18 HPV16 E7 15-mer overlapping peptides (overlapped by 10 amino acids) [36], mitogen (PMA $40 \text{ ng}/\text{mL}$ + ionomycin $4 \mu\text{g}/\text{mL}$) or medium alone (negative control) were added to a 96 well ELIIP plate (Millipore, USA) coated with anti-human interferon- γ (IFN γ) monoclonal antibodies ($15 \mu\text{g}/\text{mL}$) according to the manufacturer's protocols for ELISpot for IFN γ (MABTECH AB, Sweden). Spot numbers representing IFN γ -producing lymphocytes were analyzed as E7-CMI with a computer assisted video imaging analysis system, KS ELISPOT (Carl Zeiss Vision, Germany) [29]. The experiment was performed on three to six wells each to allow statistical analysis.

2.8. Statistical analysis

ELISpot data are presented as mean \pm standard deviation. ELISpot numbers were compared between immunization groups using Mann-Whitney *U*-test. A *p*-value of <0.05 was considered significant.

3. Results

3.1. Study population and adverse events

Participant characteristics are summarized in Table 1. All enrolled patients were Japanese women. Ten patients were enrolled in safety and dose escalation studies (Step 1) and seven additional patients were studied only at the optimized vaccination dose (4 capsules/day; Step 2). The distribution of participant HLA haplotypes was similar to that in the Japanese population [37,38]. No patient experienced serious side effects induced by GBL101c

Table 1

Baseline characteristics of the patients.

| Pt. ID | Age | Pre Tx ^a | Dose ^b | Cytology ^c | Histology | HLA-A allele |
|--------|-----|---------------------|-------------------|-----------------------|-----------|--------------|
| Step 1 | | | | | | |
| 1-1 | 40 | Untreated | 1 | HSIL, S | CIN3 | 02:06/31:01 |
| 1-2 | 42 | Laser | 2 | HSIL, S | CIN3 | 24:02/33:03 |
| 1-3 | 34 | Untreated | 2 | HSIL, S | CIN3 | 24:02/24:20 |
| 1-4 | 42 | Untreated | 2 | HSIL, S | CIN3 | 11:01/24:02 |
| 1-5 | 39 | Untreated | 4 | HSIL, M | CIN3 | 02:06/26:01 |
| 1-6 | 43 | Laser | 4 | HSIL, S | CIN3 | 02:06/26:01 |
| 1-7 | 35 | Laser | 4 | HSIL, S | CIN3 | 24:02/24:02 |
| 1-8 | 33 | Untreated | 6 | HSIL, S | CIN3 | 11:01/26:01 |
| 1-9 | 41 | Laser | 6 | HSIL, S | CIN3 | 31:01/31:01 |
| 1-10 | 37 | Untreated | 6 | HSIL, S | CIN3 | 02:01/02:01 |
| Step 2 | | | | | | |
| 2-1 | 42 | Laser, conization | 4 | HSIL, S | CIN3 | 01:01/24:02 |
| 2-2 | 36 | Laser | 4 | HSIL, S | CIN3 | 24:02/31:01 |
| 2-3 | 35 | Laser | 4 | HSIL, S | CIN3 | 24:02/24:02 |
| 2-4 | 42 | Untreated | 4 | HSIL, S | CIN3 | 24:02/26:01 |
| 2-5 | 38 | Untreated | 4 | HSIL, S | CIN3 | 11:01/26:01 |
| 2-6 | 29 | Untreated | 4 | HSIL, S | CIN3 | 02:01/31:01 |
| 2-7 | 30 | Untreated | 4 | HSIL, M | CIN3 | 02:01/24:02 |

HSIL,S: high-grade squamous intraepithelial lesion, severe dysplasia (CIN3).

HSIL,M: high-grade squamous intraepithelial lesion, moderate dysplasia (CIN2).

^a Previous treatment before the enrollment.

^b Number of capsules of GBL101c (250 mg/capsule) administered daily.

^c Both Bethesda system classification and expected diagnoses were described.

according to CTCAE. No patient was withdrawn because of adverse events or progression of their disease.

3.2. E7 specific cell-mediated immune responses (E7-CMI)

Numbers of E7-specific IFN γ -producing cells in CxLs and PBMCs were separately examined for E7-CMI [25]. Fig. 1 depicts representative pictures of our ELISpot assays. In this patient (patient 2-7), oral administration of GBL101c elicited a time-dependent increase in E7-specific IFN γ -producing CxLs but had no effect on PBMCs. A summary of E7-CMI results at baseline and week 9 is shown in Table 2 and Fig. 2. At baseline, all patients lacked E7-CMI in PBMCs while three patients had barely detectable levels of E7-CMI in CxLs. At week 9, all patients had increases in cervical E7-CMI

Table 2

E7-CMI in the cervix and peripheral blood before and after administration.

| Pt. ID | Dose | E7-CMI ^a | | | |
|--------|------|----------------------------------|----------------|---------------|----------------|
| | | Cervical lymphocyte ^b | | PBMC | |
| | | Baseline | 9 wk | Baseline | 9 wk |
| Step 1 | | | | | |
| 1-1 | 1 | 2.8 \pm 0.4 | 9.2 \pm 0.5 | 3.1 \pm 0.3 | 8.0 \pm 0.5 |
| 1-2 | 2 | 7.5 \pm 1.0 | 12.3 \pm 3.0 | 4.9 \pm 1.0 | 6.2 \pm 1.5 |
| 1-3 | 2 | 4.8 \pm 1.0 | 9.9 \pm 1.5 | 6.8 \pm 1.5 | 7.2 \pm 1.0 |
| 1-4 | 2 | 2.8 \pm 0.5 | 11.3 \pm 2.0 | 4.8 \pm 0.6 | 6.4 \pm 2.0 |
| 1-5 | 4 | 9.6 \pm 0.4 | 28.8 \pm 0.8 | 3.1 \pm 0.7 | 7.0 \pm 0.3 |
| 1-6 | 4 | 12.0 \pm 0.4 | 38.4 \pm 0.4 | 2.7 \pm 0.3 | 5.3 \pm 0.5 |
| 1-7 | 4 | 12.0 \pm 0.4 | 44.0 \pm 0.5 | 2.5 \pm 0.4 | 5.7 \pm 0.3 |
| 1-8 | 6 | 8.0 \pm 0.2 | 33.6 \pm 0.4 | 5.6 \pm 0.2 | 19.2 \pm 0.4 |
| 1-9 | 6 | 8.8 \pm 0.2 | 17.6 \pm 0.2 | 5.6 \pm 0.2 | 12.8 \pm 0.5 |
| 1-10 | 6 | 8.0 \pm 0.2 | 14.4 \pm 0.4 | 7.2 \pm 0.4 | 10.4 \pm 0.6 |
| Step 2 | | | | | |
| 2-1 | 4 | 8.3 \pm 1.6 | 18.8 \pm 1.2 | 2.3 \pm 0.4 | 6.0 \pm 0.9 |
| 2-2 | 4 | 6.8 \pm 0.7 | 33.0 \pm 1.8 | 2.3 \pm 0.4 | 6.5 \pm 0.3 |
| 2-3 | 4 | 14.3 \pm 2.5 | 40.5 \pm 1.7 | 2.5 \pm 0.5 | 6.3 \pm 0.2 |
| 2-4 | 4 | 6.0 \pm 1.1 | 21.8 \pm 2.0 | 2.0 \pm 0.3 | 6.8 \pm 0.4 |
| 2-5 | 4 | 6.8 \pm 0.7 | 24.8 \pm 2.2 | 1.8 \pm 0.4 | 6.3 \pm 0.5 |
| 2-6 | 4 | 8.3 \pm 2.0 | 14.3 \pm 1.2 | 2.3 \pm 0.5 | 6.3 \pm 0.4 |
| 2-7 | 4 | 9.0 \pm 1.1 | 36.0 \pm 3.8 | 2.0 \pm 0.4 | 5.3 \pm 0.2 |

^a Numbers of E7-specific IFN γ -producing cell ($/10^5$ cells).

^b Lymphocytes obtained from cervical lesion using the cytobrush method.

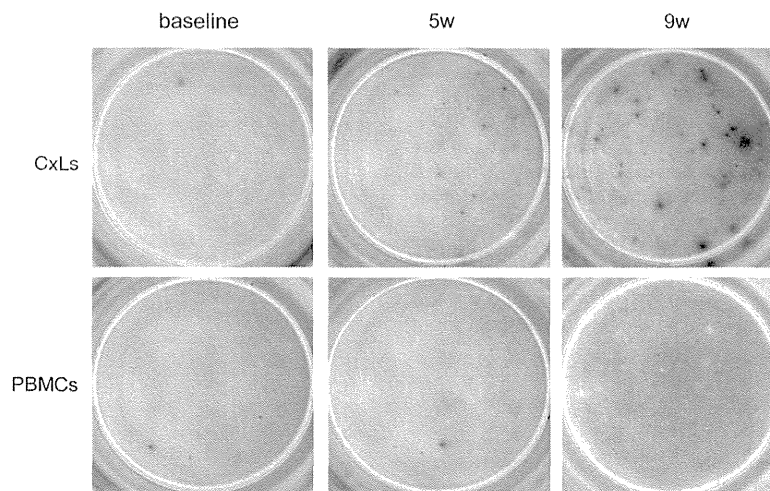


Fig. 1. Immunological response to vaccination (4 capsules/day) in a representative patient. (ELISpot assay images). CxLs (upper) and PBMCs (lower) were collected from patients 2–7 at baseline and at weeks 5 and 9. Purple dots indicated E7-specific IFN γ -producing lymphocytes. CxLs (cervical lymphocytes); PBMCs (peripheral blood mononuclear cells). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

significantly. In particular all patients taking 4 or 6 capsules/day had marked increases in cervical E7-CMI than in PBMCs. In comparison between patients of cohorts, E7-CMI at week 9 (9 wk) in patients on 4 capsules/day significantly higher than those in patients on 1, 2, or 6 capsules/day (Fig. 2). Only two of the 13 patients receiving four or six capsule doses had significant increases in E7-CMI in PBMCs. These data indicate that oral administration of GLBL101c induces predominantly mucosal E7-CMI homing to the cervical epithelium.

3.3. Clinical responses to administration

Supplementary Fig. 1 displays the clinical response of a representative patient experiencing a pathological down-grade to CIN1–2. Clinical responses at week 9 after vaccination and follow-up, cytological evaluations for all subjects are summarized in Table 3. In Step 1, the four patients taking 1–2 capsules/day had no pathological response. Two of three patients using 4 capsules/day experienced a pathological down-grade to CIN2. One of three patients on 6 capsules/day experienced a pathological down-grade to CIN2 while two had no pathological changes noted. Taken together these results with immunological responses (Fig. 2),

4 capsules/day was chosen as the optimal dose of GLBL101c and seven additional patients were enrolled at this dosage in Step 2. Combining Step 1 and 2 patients receiving 4 capsules/day, 7 of 10 patients (70%) using this regimen had a pathological down-grade to CIN2 at week 9 and one other patient (Patient ID: Step 1–5) had a pathological down-grade to CIN2 at week 12. Of the 13 patients receiving 4–6 capsules/day, nine patients (69%) with pathological down-grade to CIN2 did not require additional surgical treatment and were followed cytologically. Among the patients without additional treatment, five patients (56%) showed further cytological regression to LSIL or normal cytology by 12 months after administration. All nine patients continued to have no evidence of CIN3 when followed without intervention between 14 and 33 months. There was no correlation between clinical response and patient background characteristics (pre-treatment, baseline cytology, or HLA-A allele types).

3.4. Correlation between E7-CMI in CxLs and clinical efficacy

The number of E7-specific IFN γ -producing cells in CxLs for each patient was plotted along the y-axis as shown in Fig. 3 and divided

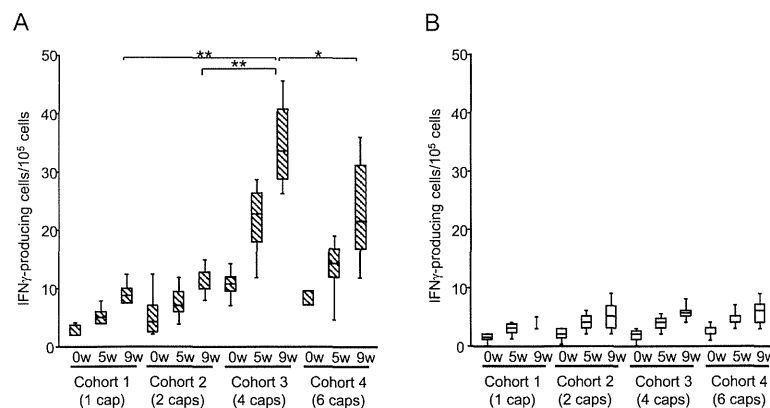


Fig. 2. Immunological responses to different dose of vaccination. (A) Stripe boxes indicate E7-CMI (E7-specific IFN γ -producing cells) in cervical lymphocytes (CxLs) for each cohort. (B) White boxes indicate E7-CMI in PBMCs for each cohort. For each cohort, E7-CMI at pre-vaccination (0 wk), weeks 5 (5 wk) and 9 (9 wk) was plotted. Each median, Inter Quartile Ranges (IQR), and maximum/minimum range is indicated using horizontal lines, boxes, and vertical length lines, respectively. Asterisks indicate those comparisons with statistical significance (*: $p < 0.01$, **: $p < 0.001$).

Table 3
Clinical efficacy of GLBL101c oral administration.

| Pt. ID | Dose | Histology(9 wk) | Cytology ^a (9 wk) | Tx ^b (10–12 wk) | Follow-up | |
|--------|------|-----------------|------------------------------|----------------------------|-----------|-----------|
| | | | | | 6 months | 12 months |
| Step 1 | | | | | | |
| 1-1 | 1 | CIN3 | HSIL, S | Conization | | |
| 1-2 | 2 | CIN3 | HSIL, S | Conization | | |
| 1-3 | 2 | CIN3 | HSIL, S | Conization | | |
| 1-4 | 2 | CIN3 | HSIL, S | Conization | | |
| 1-5 | 4 | CIN3 | HSIL, M | (-) | HSIL, M | LSIL |
| 1-6 | 4 | CIN2 | HSIL, S | (-) | HSIL, M | HSIL, M |
| 1-7 | 4 | CIN2 | HSIL, M | (-) | LSIL | NILM |
| 1-8 | 6 | CIN2 | HSIL, M | (-) | HSIL, M | NILM |
| 1-9 | 6 | CIN3 | HSIL, S | Laser | | |
| 1-10 | 6 | CIN3 | HSIL, S | Laser | | |
| Step 2 | | | | | | |
| 2-1 | 4 | CIN3 | HSIL, M | Laser | | |
| 2-2 | 4 | CIN2 | HSIL, M | (-) | HSIL, M | HSIL, M |
| 2-3 | 4 | CIN2 | HSIL, S | (-) | HSIL, M | HSIL, M |
| 2-4 | 4 | CIN2 | HSIL, M | (-) | HSIL, M | HSIL, M |
| 2-5 | 4 | CIN2 | HSIL, S | (-) | LSIL | NILM |
| 2-6 | 4 | CIN3 | HSIL, S | Laser | | |
| 2-7 | 4 | CIN2 | HSIL, M | (-) | LSIL | LSIL |

HSIL,S: high-grade squamous intraepithelial lesion, severe dysplasia (CIN3).

HSIL,M: high-grade squamous intraepithelial lesion, moderate dysplasia (CIN2).

LSIL: low-grade squamous intraepithelial lesion.

NILM: negative for intraepithelial lesion or malignancy.

^a Both Bethesda system classification and expected diagnoses are presented.

^b Surgical treatment methods at week 10–12. (-): The patients with no surgical treatment were evaluated with standard cervical cytology.

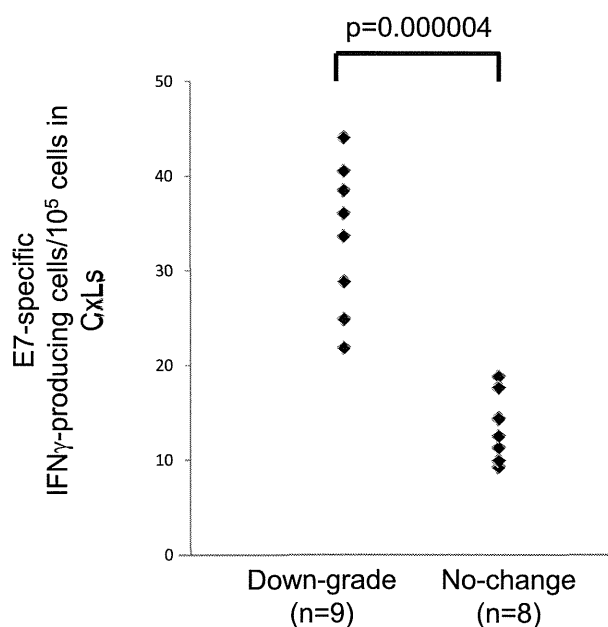


Fig. 3. Correlation of E7-CMI in CxLs with pathological responses. E7-CMI (E7-specific IFN γ -producing cells) at week 9 was compared between pathological down-grade ($n=9$) and no change ($n=8$) groups. The cervical E7-CMI of down-graded patients increased significantly more than that of patients who had no change (Mann-Whitney U -test: $p=0.000004$). The ROC curve indicated a cut-off value = 21.8 cells/ 10^5 cells and AUC = 0.994.

into groups, patients experiencing a pathological down-grade ($n=9$) and those who had no pathological change ($n=8$). E7-CMI in the CxLs of patients experiencing a pathological down-grade was clearly higher than that among patients with no pathological change ($p=0.000004$). ROC analysis of E7-CMI in CxLs indicated a cut-off value of 21.8 IFN γ -producing cells/ 10^5 cells (AUC = 0.994)

for pathological regression and a sensitivity and specificity using this cut-off value of 94.5% and 99.2%, respectively (data not shown).

4. Discussion

Our study is the first report demonstrating that oral vaccination promotes clinical response in a mucosal intraepithelial neoplasm by inducing vaccine antigen-specific mucosal CMI. Interestingly, the clinical responses to GLBL101c correlated directly with mucosal E7-CMI within the uterine cervix but systemic E7-CMI was not generally elicited. This suggests strongly that mucosal effector T cells are induced by oral administration of the Lactobacillus-based HPV vaccine at intestinal mucosal inductive sites, eg Peyer's patches, and that these cells home to the cervical mucosa to direct the immunological microenvironment to type1 immune responses to HPV-related intraepithelial neoplasia. Mucosal T cells possessing E7-CMI and educated in the gut are thought to enter the peripheral circulation to home to the cervix. However, E7-CMI in the peripheral blood was negligible in our ELISpot data regardless of GLBL101c dose. The concentration of E7-specific mucosal T cells in the peripheral blood may have been too low to be detected by ELISpot assay due to dilution of the lymphocytes in the circulation. In contrast, the integrin $\beta 7$ + mucosal T cells possessing E7-CMI accumulate and are retained in the mucosal epithelium via interactions of integrin $\alpha E\beta 7$ with E-cadherin expressed in the epithelium [39].

In this trial, the pathological down-grade to CIN2 in response to a 4 capsules/day GLBL101c regimen was 80%. In Japan, CIN3 is treated surgically whereas CIN2 is generally monitored without surgical intervention by gynecologic oncologist. Therefore Japanese pathologists are routinely required to discriminate CIN2 from CIN3 in an effort to direct clinical management. Pathological down-grading continues to have important clinical implications in Japan. Data from several previous clinical trials of HPV therapeutic vaccines estimate a spontaneous regression rate from CIN3 to CIN2 of 10% based on data of non-intervention cohort studies or the placebo-arm of randomized clinical trials [13,31]. We purposely delayed repeat specimen collection until week 9, because the biopsy procedure can itself promote spontaneous regression [40]. Although

the best clinical response during 9 weeks of this study was a down-grade to CIN2, the observed clinical response rate was significantly higher than either the estimated or described rate of spontaneous down-grading to CIN2. Notably, 9 of 13 (69%) patients with pathological responses to 4–6 capsules/day remained free of CIN3 for 14–33 months after administration. Moreover, five of them had further cytological regression to LSIL or normal cytology (NILM) at 12 months after the last administration.

L. casei is one of the most commonly consumed bacterial species worldwide and its safety is well-demonstrated. No adverse event greater than grade 2 has been reported in any exposed patient in prior studies. Several studies on immunization with Lactobacillus-based vaccines have demonstrated an induction of systemic E7-CMI and regression of subcutaneous TC-1-induced tumors [26–28]. However, they have neither provided an insight into mucosal T cell responses to oral vaccination nor into the anti-tumor effects on mucosal intraepithelial neoplasms. While this could represent a difference between humans and mice, the mucosal specificity of the response in this human trial remains a useful attribute for further vaccine development. We previously reported a marked induction of mucosal T cells possessing E7-CMI within intestinal mucosae after oral administration of *L. casei* expressing HPV16 E7 in mice [29] and developed this clinical trial in response.

In this study, one of three patients on 6 capsules/day had clinical response while all patients using 4 capsules/day had a down-grade to CIN2 by week 12. Furthermore, E7-CMI in patients on 4 capsules/day was significantly higher than that in patients on 6 capsules/day. This may be the result of small sample size and will need to be studied further. Although we cannot conclusively state that 4 capsules/day is the optimal dose, we can state that this dose is both safe and effective.

Our GLBL101c regimen was associated with pathological down-grading from CIN3 to CIN2. Although this changes clinical management in Japan, changes in worldwide diagnostic guidelines mask the therapeutic clinical benefit of our regimen because CIN2 and CIN3 are grouped and both are treated surgically. Nevertheless, in this study, nine patients who experienced a down-grade to CIN2 were followed without surgery and remained free of CIN3. These patients may benefit from oral GLBL101c administration. Future randomized placebo-controlled studies to evaluate clinical efficacy of oral vaccination with GLBL101c should therefore include follow-up time points of at least 4–6 months after completion of the regimen.

5. Conclusion

Oral administration of a Lactobacillus-based HPV therapeutic vaccine succeeded in inducing mucosal but not systemic E7-CMI. This study is the first to report a correlation between mucosal CMI and clinical response of an immunotherapy in human mucosal neoplasia. This vaccine strategy may be a novel HPV-targeting immunotherapy for cervical cancer involving the induction of E7-specific mucosal immunity. Furthermore, the oral administration of Lactobacillus-based vaccine may extend to other diseases that develop at mucosal sites including bowel, bronchial, and oropharyngeal epitheliae.

This clinical trial is registered to UMIN-CTR which is accepted by ICMJE.

Clinical registration ID: UMIN000001686 (2009/02/06).

IRB approval No.: P9002144-11X.

The director of this study is K.K. This study was designed by K.K, Y.O, and T.F. K.K and K.A wrote the main manuscript text and prepared all figures. K.K, K.A, S.K, A.T, K.T, A.Y, H.N, K.N, T.A, O.W-H, and K.O collected samples from patients. K.A, S.K, A.T, K.T, K.N,

T.Y, and H.N performed these experiments. T.Y and T.S provided GLBL101c. All authors reviewed the manuscript.

Acknowledgments

We are grateful to Dr. D. J. Schust for careful and critical editing of the manuscript, Dr. E. Z. Drobnis for her advice on statistical approaches, and Dr. A. Tachikawa for her technical advice on ELISpot assays. This work was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan (KK) and for Comprehensive Strategy for Practical Medical Technology, by a cancer research grant from the Ministry of Education, Culture, Sports, Science and Technology (KK) of Japan, by a grant from Kanzawa Medical Research Foundation (KK), TOKYO IGAKUKAI (KK) and by a grant from the Okinawa New Industry Creation Project (TS).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2014.09.020>.

References

- [1] zur Hausen H. Papillomavirus and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002;2(5):342–50.
- [2] Dunne EF, Unqer ER, Sternberg M, McQuillan G, Swan DC, Patel SS, et al. Prevalence of HPV infection among females in the United States. *J Am Med Assoc* 2007;297(8):813–9.
- [3] Bosch FX, de Sanjose S. Human papillomavirus and cervical cancer—burden and assessment of causality. *J Natl Cancer Inst Monogr* 2003;31:3–13.
- [4] Masumoto N, Fujii T, Ishikawa M, Mukai M, Ono A, Iwata T, et al. Dominant human papillomavirus 16 infection in cervical neoplasia in young Japanese women: study of 881 outpatients. *Gynecol Oncol* 2004;94(2):509–14.
- [5] Miura S, Matsumoto K, Okai A, Satoh T, Tsunoda H, Yasugi T, et al. Do we need a different strategy for HPV screening and vaccination in East Asia? *Int J Cancer* 2006;119(11):2713–5.
- [6] Koutsky LA, Ault KA, Wheeler CM, Brown DR, Barr E, Alvarez FB, et al. A controlled trial of a human papillomavirus type 16 vaccine. *N Engl J Med* 2002;347(21):1645–51.
- [7] Paavonen J, Naud P, Salmérón J, Wheeler CM, Chow SN, Apter D, et al. Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuncted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomized study in young women. *Lancet* 2009;374(9686):301–14.
- [8] Muñoz N, Kjaer SK, Sigurdsson K, Iversen OE, Hernandez-Avila M, Wheeler CM, et al. Impact of Human papillomavirus (HPV)-6/11/16/18 vaccine on all HPV-associated genital diseases in young women. *J Natl Cancer Inst* 2010;102(5):325–39.
- [9] Villa LL, Costa RL, Petta CA, Andrade RP, Paavonen J, Iversen OE, et al. High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up. *Br J Cancer* 2006;95(11):1459–66.
- [10] Harper DM, Franco EL, Wheeler CM, Moscicki AB, Romanowski B, Roteli-Martins CM, et al. Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. *Lancet* 2006;367(9518):1247–55.
- [11] Kawana K, Yasugi T, Taketani Y. Human papillomavirus vaccines: current issues and future: review. *Indian J Med Res* 2009;130(3):341–7.
- [12] Kanodia S, Da Silva DM, Kast WM. Recent advances in strategies for immunotherapy of human papillomavirus-induced lesions. *Int J Cancer* 2008;122(2):247–59.
- [13] Einstein MH, Kadish AS, Burk RD, Kim MY, Wadler S, Streicher H, et al. Heat shock fusion protein-based immunotherapy for treatment of cervical intraepithelial neoplasia III. *Gynecol Oncol* 2007;106(3):453–60.
- [14] Roman LD, Wilczynski S, Muterspach LI, Burnett AF, O'Meara A, Brinkman JA, et al. A phase II study of Hsp-7 (SGN-00101) in women with high-grade cervical intraepithelial neoplasia. *Gynecol Oncol* 2007;106(3):558–66.
- [15] Kaufmann AM, Nieland JD, Jochmus I, Baur S, Frieze K, Gabelsberger J, et al. Vaccination trial with HPV16 L1E7 chimeric virus-like particles in women suffering from high grade cervical intraepithelial neoplasia (CIN 2/3). *Int J Cancer* 2007;121(12):2794–800.
- [16] Davidson EJ, Boswell CM, Sehr P, Pawlita M, Tomlinson AE, McVey RJ, et al. Immunological and clinical responses in women with vulvar intraepithelial neoplasia vaccinated with a vaccinia virus encoding human papillomavirus 16/18 oncoproteins. *Cancer Res* 2003;63(18):6032–41.
- [17] Fiander AN, Tristram AJ, Davidson EJ, Tomlinson AE, Man S, Baldwin PJ, et al. Prime-boost vaccination strategy in women with high-grade, noncervical

- anogenital intraepithelial neoplasia: clinical results from a multicenter phase II trial. *Int J Gynecol Cancer* 2006;16(3):1075–81.
- [18] García-Hernández E, González-Sánchez JL, Andrade-Manzano A, Contreras ML, Padilla S, Guzmán CC, et al. Regression of papilloma high-grade lesions (CIN 2 and CIN 3) is stimulated by therapeutic vaccination with MVA E2 recombinant vaccine. *Cancer Gene Ther* 2006;13(6):592–7.
- [19] Garcia F, Petry KU, Muderspach L, Gold MA, Braly P, Crum CP, et al. ZYC101a for treatment of high-grade cervical intraepithelial neoplasia: a randomized controlled trial. *Obstet Gynecol* 2004;103(2):317–26.
- [20] Kenter CG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med* 2009;361(19):1838–47.
- [21] Brun JL, Dalstein V, Leveque J, Mathevet P, Raulic P, Baldauf JJ, et al. Regression of high-grade cervical intraepithelial neoplasia with TG4001 targeted immunotherapy. *Am J Obstet Gynecol* 2011;204(2):169 (e1–8).
- [22] Bagarazzi ML, Yan J, Morrow MP, Shen X, Parker RL, Lee JC, et al. Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses. *Sci Transl Med* 2012;4(155)(155ra138).
- [23] Trimble CL, Peng S, Kos F, Gravitt P, Viscidi R, Sugar E, et al. A phase I trial of a human papillomavirus DNA vaccine for HPV16+ cervical intraepithelial neoplasia 2/3. *Clin Cancer Res* 2009;15(1):361–7.
- [24] Gorfú G, Nieves JR, Ley K. Role of beta7 integrins in intestinal lymphocyte homing and retention. *Curr Mol Med* 2009;9(7):836–50.
- [25] Kojima S, Kawana K, Fujii T, Yokoyama T, Miura S, Tomio K, et al. Characterization of gut-derived intraepithelial lymphocyte (IEL) residing in human papillomavirus (HPV)-infected intraepithelial neoplastic lesions. *Am J Reprod Immunol* 2011;66(5):435–43.
- [26] Bermúdez-Humarán LG, Cortes-Perez NG, Lefèvre F, Guimarães V, Rabot S, Alcocer-Gonzalez JM, et al. A novel mucosal vaccine based on live Lactococci expressing E7 antigen and IL-12 induces systemic and mucosal immune responses and protects mice against human papillomavirus type 16-induced tumors. *J Immunol* 2005;175(11):7297–302.
- [27] Cortes-Perez NG, Lefevre F, Corthier G, Adel-Patient K, Langella P, Bermúdez-Humarán LG. Influence of the route of immunization and the nature of the bacterial vector on immunogenicity of mucosal vaccines based on lactic acid bacteria. *Vaccine* 2007;25(36):6581–8.
- [28] Poo H, Pyo HM, Lee TY, Yoon SW, Lee JS, Kim CJ, et al. Oral administration of human papillomavirus type 16 E7 displayed on *Lactobacillus casei* induces E7-specific antitumor effects in C57/BL6 mice. *Int J Cancer* 2006;119(7):1702–9.
- [29] Adachi K, Kawana K, Yokoyama T, Fujii T, Tomio A, Miura S, et al. Oral immunization with *Lactobacillus casei* vaccine expressing human papillomavirus (HPV) type 16 E7 is an effective strategy to induce mucosal cytotoxic lymphocyte against HPV16 E7. *Vaccine* 2010;28(16):2810–7.
- [30] Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlée F, Hildesheim A, et al. Improved amplification of genital human papillomaviruses. *J Clin Microbiol* 2000;38(1):357–61.
- [31] Follen M, Atkinson EN, Schottenfeld D, Malpica A, West L, Lippman S, et al. A randomized clinical trial of 4-hydroxyphenylretinamide for high-grade squamous intraepithelial lesions of the cervix. *Clin Can Res* 2001;7(11):3356–65.
- [32] Keefe KA, Schell MJ, Brewer C, McHale M, Brewster W, Chapman JA, et al. A randomized, double blind, Phase III trial using oral beta-carotene supplementation for women with high-grade cervical intraepithelial neoplasia. *Cancer Epidemiol Biomark Prev* 2001;10(10):1029–35.
- [33] van der Burg SH, Kwappenberg KM, O'Neill T, Brandt RM, Melief CJ, Hickling JK, et al. Pre-clinical safety and efficacy of TA-CIN, a recombinant HPV16 L2E6E7 fusion protein vaccine, in homologous and heterologous prime-boost regimens. *Vaccine* 2001;19(27):3652–60.
- [34] Prakash M, Patterson S, Kapembwa MS. Evaluation of the cervical cytobrush sampling technique for the preparation of CD45+ mononuclear cells from the human cervix. *J Immunol Methods* 2001;258(1–2):37–46.
- [35] Buckley CH, Butler EB, Fox H. Vulvar intraepithelial neoplasia and microinvasive carcinoma of the vulva. *J Clin Pathol* 1984;37(11):1201–11.
- [36] Peng S, Trimble C, Wu L, Pardoll D, Roden R, Hung CF, et al. HLA-DQB1*02-restricted HPV-16 E7 peptide-specific CD4+ T-cell immune responses correlate with regression of HPV-16-associated high-grade squamous intraepithelial lesions. *Clin Cancer Res* 2007;13(8):2479–87.
- [37] Trimble CL, Piantadosi S, Gravitt P, Ronnett B, Pizer E, Elko A, et al. Spontaneous regression of high-grade cervical dysplasia: effects of human papillomavirus type and HLA phenotype. *Clin Cancer Res* 2005;11(13):4717–23.
- [38] Saito M, Okubo M, Hirata R, Takeda S, Maeda H. Association of human leukocyte antigen and T cell message with human papillomavirus 16-positive cervical neoplasia in Japanese women. *Int J Gynecol Cancer* 2007;17(6):1314–21.
- [39] Ericsson A, Svensson M, Arya A, Agace WW. CCL25/CCR9 promotes the induction and function of CD103 on intestinal intraepithelial lymphocytes. *Eur J Immunol* 2004;34:2720–9.
- [40] Ostor A. The natural history of cervical intraepithelial neoplasia: a critical review. *Int J Gynecol Pathol* 1993;12:186–92.

Characterization of Novel Transcripts of Human Papillomavirus Type 16 Using Cap Analysis Gene Expression Technology

Ayumi Taguchi,^a Kazunori Nagasaka,^a Kei Kawana,^a Kosuke Hashimoto,^b Rika Kusumoto-Matsuo,^c Charles Plessy,^b Miranda Thomas,^d Hiroe Nakamura,^a Alessandro Bonetti,^b Katsutoshi Oda,^a Iwao Kukimoto,^c Piero Carninci,^b Lawrence Banks,^d Yutaka Osuga,^a Tomoyuki Fujii^a

Department of Obstetrics and Gynecology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan^a; RIKEN Center for Life Science Technologies, Division of Genomic Technologies, Yokohama, Japan^b; Pathogen Genomic Center, National Institute of Infectious Diseases, Tokyo, Japan^c; International Centre for Genetic Engineering and Biotechnology, Trieste, Italy^d

We have performed cap-analysis gene expression (CAGE) sequencing to identify the regulatory networks that orchestrate genome-wide transcription in human papillomavirus type 16 (HPV16)-positive cervical cell lines of different grades: W12E, SiHa, and CaSki. Additionally, a cervical intraepithelial neoplasia grade 1 (CIN1) lesion was assessed for identifying the transcriptome expression profile. Here we have precisely identified a novel antisense noncoding viral transcript in HPV16. In conclusion, CAGE sequencing should pave the way for understanding a diversity of viral transcript expression.

Cervical cancer is the third most common female cancer worldwide, with 530,000 new cases and 275,000 deaths annually (1), and infection by one of a subset of high-risk human papillomaviruses (HPVs) is a prerequisite for its development (2).

A number of elegant studies on the HPV type 16 (HPV16) transcriptome (3–10) have shown that transcription in all HPVs occurs unidirectionally from at least two promoters and involves multiple splice sites. The major promoters in HPV16 are the early promoter (p97), located in the locus control region (LCR), and the differentiation-inducible promoter (p670), with additional promoters in the LCR and within E4 and E5, potentially generating late transcripts. HPV transcription is polycistronic, undergoing differential splicing to produce numerous viral mRNAs (at least 13 transcripts from 8 HPV16 genes in W12E cells [11]). Previous analyses considered only coding RNA, and it is possible that new transcription start sites (TSSs) are obscured by prominent promoters. Therefore, we have used cap analysis gene expression (CAGE) for high-throughput transcriptome analysis (12–16). CAGE specifically determines transcriptional start sites (TSS) genome wide, by capping 5' ends of RNA transcripts, reverse transcribing them, and mapping cDNAs to the reference genome to identify TSSs (17–20).

To assess all potential TSSs in HPV16-infected cell lines, we performed CAGE analysis using cervical carcinoma-derived CaSki and SiHa cells (21, 22), cervical intraepithelial neoplasia grade 1 (CIN1)-derived W12E (20863) cells, containing episomal copies of HPV16 (3, 23) (3T3 feeder cells removed by trypsinization), and a biopsy specimen from a CIN1 cervical lesion (approved by the Institutional Review Board, University of Tokyo Hospital). From each sample, 5 μ g RNA was extracted using a miRNeasy kit (Qiagen). RNA quality was assessed by the use of a Bioanalyzer (Agilent) and standardized at an RNA integrity number (RIN) of >7.0 . Quantitation by NanoDrop analysis confirmed that the A_{260}/A_{290} and A_{260}/A_{230} ratios were >1.7 .

First-strand cDNAs were transcribed to the 5' end of capped RNAs and attached to CAGE “bar code” tags (Table 1), and the sequenced CAGE tags were mapped to the HPV16 genome and the human hg19 genomes using BWA software (v0.5.9), discarding ribosomal or non-A/C/G/T base-containing RNAs (24).

CAGE tags were normalized by the total number of tags per sample mapped to the human genome and are indicated as tags per million (TPM) (25). For HPV16 genes, CAGE tag 5' coordinates were input for Paraclu clustering, with these parameters: (i) a minimum of 5 tags/cluster, (ii) (maximum density/baseline density) ≥ 2 , and (iii) 100-bp maximal cluster length (18).

HPV16 transcriptomes differ between carcinoma- and CIN1-derived cells. Viral gene expression was high in W12E cells and CIN1 clinical samples (Fig. 1C) but was low in CaSki and SiHa cells (Table 1). To quantitatively visualize CAGE-tagged HPV16 genes from each cell line, we compared the data to the HPV16 reference genome (NCBI GI no. 333031). We found 25 positive-strand and 4 negative-strand tag clusters (TCs [regions with >0.5 TPM/sample]) in W12E cells (Fig. 1A), and only 1 positive-strand TC in CaSki and SiHa cells (Fig. 1B), corresponding to the p97 promoters. In W12E cells and a CIN1 clinical sample, numerous additional TSSs span the HPV16 genome, suggesting that precancerous cells show diversity and abundance of viral gene expression that are lost upon viral genome integration and disease progression, although investigation of W12 derivatives with integrated HPV16 and of additional clinical samples is needed to confirm this.

The six most highly expressed TCs in each cell line are compared in Fig. 2. The six TCs most frequently found in W12E cells originate from nucleotide (nt) 90 to 97, nt 1125 to 1149, nt 2017 to

Received 1 December 2014 Accepted 2 December 2014

Accepted manuscript posted online 10 December 2014

Citation Taguchi A, Nagasaka K, Kawana K, Hashimoto K, Kusumoto-Matsuo R, Plessy C, Thomas M, Nakamura H, Bonetti A, Oda K, Kukimoto I, Carninci P, Banks L, Osuga Y, Fujii T. 2015. Characterization of novel transcripts of human papillomavirus type 16 using cap analysis gene expression technology. *J Virol* 89:2448–2452. doi:10.1128/JVI.03433-14.

Editor: M. J. Imperiale

Address correspondence to Kazunori Nagasaka, nagasakak-ky@umin.ac.jp, or Kei Kawana, kkawana-ky@umin.org.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.03433-14