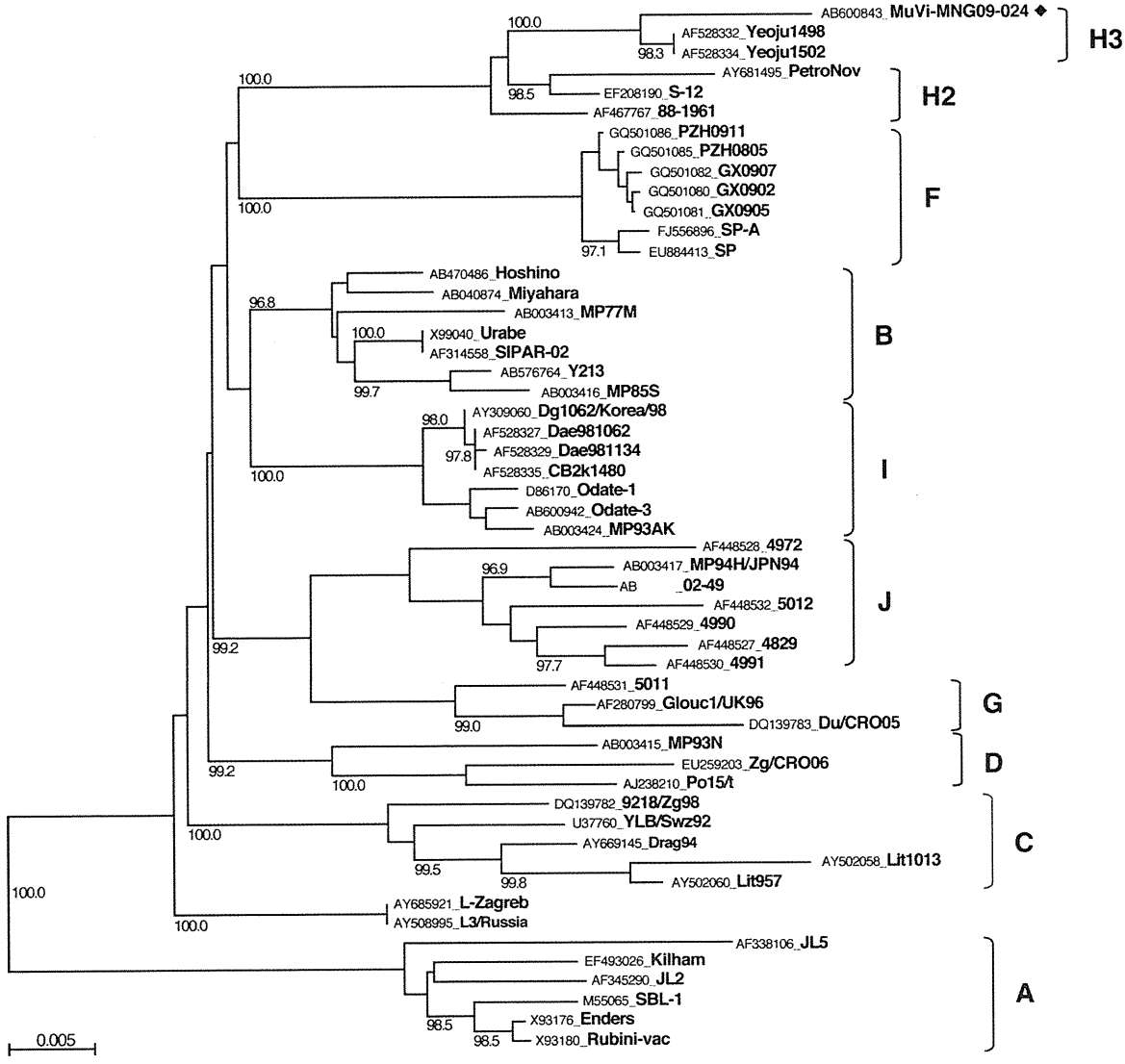

FIG. 4. Phylogenetic tree based on HN gene sequences. ◆, Mumps virus strains obtained in the present study.

Primarily, phylogenetic analyses results based on F and HN genes are consistent with the SH gene. However, when there would be discrepancies between the phylogenetic results based on the SH gene and that based on F or HN genes or the whole-genome, and latter results were consistent with each other, the latter results should be adopted. From these results, we propose that F or HN gene sequences should be used in combination with SH gene data for confirming a new MuV genotype. By taking this additional information into the criteria for genotyping, it would be possible to reflect the antigenicities of MuVs to the phylogenetic results and to clarify the phylogenetic positions of outliers such as the strains RW(X63708), Tay-UK50(AF142774), and UK02-19(AY380077) (23). In order to establish the new criteria, we need to accumulate more F or HN gene sequence data of existing MuV strains.

Molecular phylogenetic analyses show that the Mongolia MuV sequences form a single cluster, and that these viruses are most closely related to a cluster of genotype H viruses, such as Japanese isolate (SA475/JPN97, isolated in 1997) (37), Korean isolates (Yeoju1498, Yeoju1502, Yeoju1503, and Yeoju1504, isolated in 1999) (18, 19) and Swiss isolates (776273SHG and 776274SHG, isolated in 1999 and 2000) (38). These strains were all isolated within a relatively short time period, from 1997 to 2000, and their SH gene sequences are almost homologous at the amino acid level (Table 2). The alignment of their SH gene nucleotide sequences suggests that SA475/JPN97 is phylogenetically closest to the ancestral virus of all of these viruses and that Mongolia viruses could be derived from the same ancestral virus because this cluster of viruses share the characteristic two conserved amino acid sequences at positions 19 and 26 in the SH gene (18) (Table 2). This amino acid motif characterizes this cluster of viruses and is never observed among other genotype H viruses. For these reasons and based on the results of phylogenetic analyses on F and HN genes, these viruses should be classified into a unique subgenotype, H3. The other two subgenotypes, H1 and H2, also have characteristic amino acid motifs within the SH region

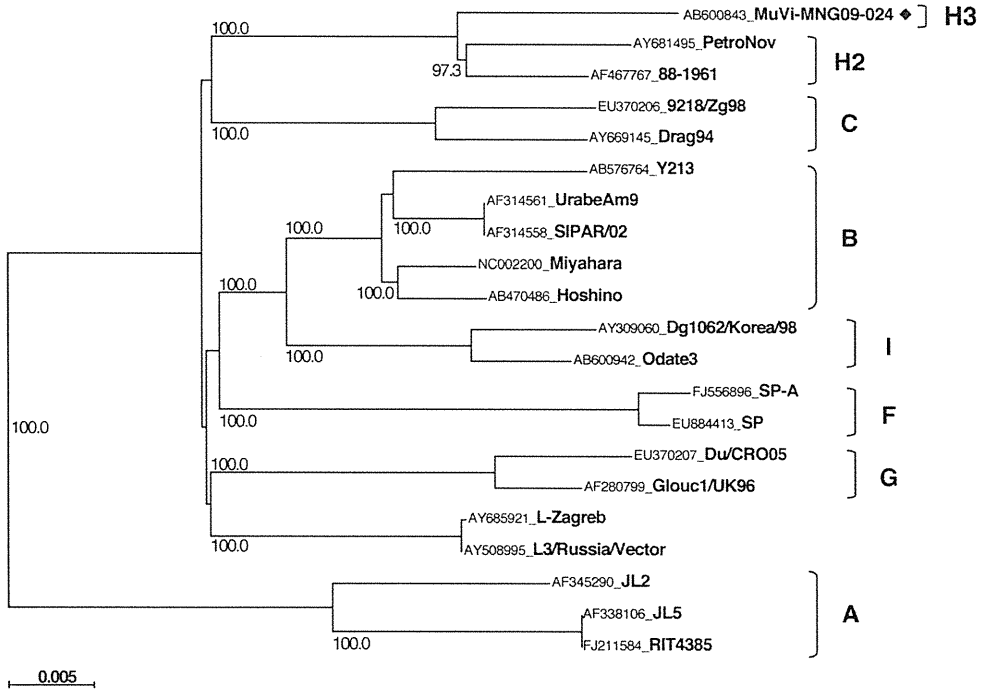

FIG. 5. Phylogenetic tree based on whole-genome sequences. ◆, Mumps virus strains obtained in the present study.

(Table 2). For instance, H1 viruses share a phenylalanine and alanine at positions 24 and 36. H2 viruses have no specific motif, other than a threonine residue at position 42, which is common in all genotype H viruses (Table 2). Meanwhile, all H2 viruses except Be1/UK88 strain share a histidine at position 9, which is also common in H1 and H2 viruses. These amino acid sequence properties suggest that subgenotype H2 may be an ancestral group of genotype H viruses and that the Be1/UK88 strain may be phylogenetically closest to an ancestral virus of genotype H.

Genotype H viruses mainly circulated in the western hemisphere (3, 5, 6, 20). However, since both the ancestral virus (SA475/JPN97) and the highly differentiated progenies (the Mongolian MuVs) were isolated from East Asia, genotype H3 viruses may have been indigenous to this area for more than 10 years. Although 776273SHG and 776274SHG were isolated in Switzerland, they might originate from the Asian origin virus because of their phylogenetic position and the time at which the viruses were isolated (38). In the present study, we did not detect any other genotypes, such as F, which is the dominant genotype in China. A major reason for this may be the restricted time period and region for collecting specimens in this study. Our samples were collected within 6 months from the patients residing in Ulaanbaatar and surrounding areas. Accordingly, it may be possible to detect other genotypes if we extended our study to cover larger areas in Mongolia for longer periods. In any case, this is the first report of MuVs circulated in Mongolia. Molecular epidemiological research on MuVs in this area has just started.

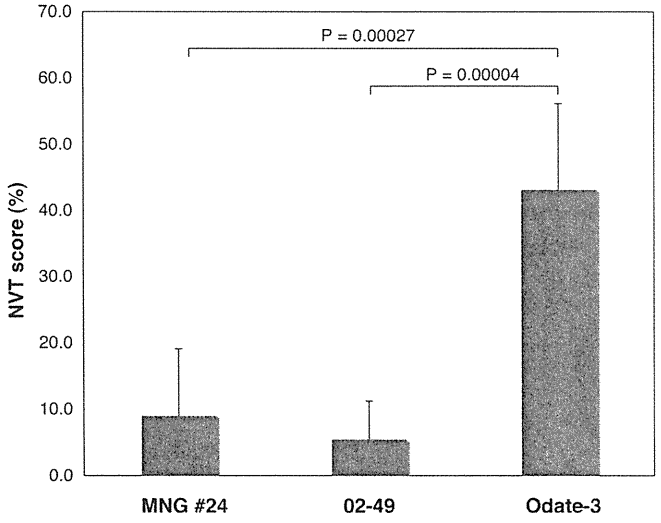

FIG. 6. Neurovirulence of MuV isolates in newborn rats. Each bar represents the average neurovirulence score with the corresponding standard deviation from six to seven animals.

Several studies have suggested that there may be an association between several genotypes (C, D, H, I, or J) and neurovirulence (34, 36, 38). Meanwhile, Utz has reported that there is no statistical relationship between neurovirulence and genotype H (38). In order to clarify this ambiguity, we evaluated the neurovirulence of the Mongolia isolate by using a newborn rat system. As a result, the Mongolia isolate was found to only exhibit mild neuropathogenic property comparable with genotype J isolate, 02-49 strain. In contrast, genotype I Japanese isolate Odate-3 shows the highest neuropathogenicity among the three isolates, and faithfully reflects its highly neurovirulent properties in humans and in marmosets. Although these three strains are all classified in genotypes possibly associated with neurovirulence, their pathogenicities in rats are divided. In addition, genotype I strains isolated in Korea were not neces-

sarily highly pathogenic (18). These facts suggest that there is no association between genotypes and neurovirulence.

We describe here the molecular epidemiology of the MuVs circulating in Mongolia. It is important to continuously investigate genotype distribution in this area in order to develop an understanding of not merely the domestic but the global epidemiology of mumps infection.

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Prevalence and type distribution of human papillomavirus in healthy Japanese women aged 20 to 25 years old enrolled in a clinical study

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Efficacy, immunogenicity and tolerability of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine were evaluated in Japanese women aged 20–25 years, for which results have been reported previously. We analyzed the baseline data from that study and report the prevalence rates of HPV infection in young healthy Japanese women. One thousand and the forty Japanese women aged 20–25 years were enrolled in a phase II, double-blind, controlled, randomized, multicenter study. At study entry, cervical specimens were collected from the women and tested by line probe assay for 25 HPV-types and by HPV-16/18-specific polymerase chain reaction. The most frequently detected HPV-type in baseline cervical specimens was HPV-52 (8.1%), followed by HPV-16 (6.5%), HPV-51 (4.5%), HPV-18 (4.0%) and HPV-31 (3.8%). The proportion of HPV DNA-positive women increased with severity of cytological abnormalities: 26.1% (237/908) in normal cytology, 93.3% (70/75) in low-grade squamous intraepithelial lesion and 100% (7/7) in high-grade squamous intraepithelial lesion. The relative contribution of HPV-16 and HPV-18 was 4.1 and 3.0% for normal cytology cases, and 20.0 and 16.0% in low-grade squamous intraepithelial lesion, respectively. HPV-16 was found in four of seven high-grade squamous intraepithelial lesion cases (57.1%) and five of the six cervical intraepithelial neoplasia 2+ cases (83.3%). Multiple and single HPV infections were observed in 13.5% (140/1039) and 20.7% (215/1039) of all women, respectively. The HPV prevalence rates in Japanese women aged 20–25 years underline the importance of HPV vaccination at a young age and this report should be useful for monitoring changes in HPV prevalence after widespread HPV vaccination in Japanese women. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.01878.x, 2011)

Persistent infection with HPV is the necessary cause for developing cervical cancer,[1] the second most common cancer in women worldwide.[2] HPV is a common sexually transmitted infection. Population-based studies suggest that up to as much as 80% of sexually active women are exposed to at least one genital HPV type in their lifetime.[3] While HPV infection occurrence is highest in young sexually active women, a relatively high HPV prevalence has also been detected in postmenopausal women. Infection at this later stage may be due to reactivation of earlier acquired infections or infection from new sexual partners later in life.[4] At least 14 oncogenic HPV types (high-risk HPV) have been causally linked to cervical cancer.[5] HPV-16 and HPV-18 are the most prevalent and account for more than 70% of all invasive cervical cancers worldwide; HPV-31 and HPV-45 are responsible for an additional 10% of cases[6,7] followed by HPV-33, HPV-35, HPV-52 and HPV-58.[6,7] HPV vaccines are now licensed in more than 100 countries, coinciding with large-scale national and regional immunization programs aimed at young adolescent girls.[8] Since infection with HPV may occur throughout the lifetime of a sexually active woman, it is important that vaccination induces a strong, sustained antibody response to ensure long-term protection.[9]

Currently, there are two HPV vaccines available in the world: a bivalent (Cervarix®, GlaxoSmithKline Biologicals) and a quadrivalent (Gardasil®, Merck). The bivalent vaccine is an AS04-adjuvanted vaccine specifically targeting HPV-16 and -18 types, while the quadrivalent vaccine is an aluminium-adjuvanted vaccine specifically targeting HPV-6, -11, -16 and -18 types. Both vaccines are immunogenic, generally well-tolerated, with clinically acceptable safety profiles.[10–13] Prevention by both vaccines of CIN2+ lesions caused by HPV-16 and -18 was >90% in women aged 15–25 years old and negative for these HPV-types at study entry.[11,14,15] Furthermore, excisional therapies for CIN2+ are reduced by approximately 70% for the bivalent vaccine[16] and 40% for the quadrivalent.[17] Sustained efficacy of both vaccines has been documented for 5 years or more.[11,15,16,18,19] However, it may take 5–10 years after the start of a vaccination program before significant reductions in the incidence of cervical cancer will be apparent.[20]

In Japan, cervical cancer ranks approximately 7th in women overall (incidence rate of 13.6 per 100 000) and 2nd in women aged 15–44 years (incidence rate of 12.0 per 100 000).[21,22] It is estimated that 15 000 women are diagnosed with cervical cancer yearly, leading to approximately 3500 deaths.[21–23] These figures include approximately 2000 new cases and 200 deaths that are estimated to occur every year in Japanese women in their twenties and thirties.[23,24] HPV is currently one of the least known sexually transmitted infections in Japan, resulting in a lower level of public knowledge of the risks of HPV and cervical cancer.[25] HPV-16 and HPV-18 are the most frequently identified HPV-types in invasive cervical cancers in Japan (67.1%), and HPV-52 and HPV-58 appear to be the next most common types accounting for 11.5% of cervical cancers.[26] Prevalence of HPV infection in the Japanese population has been mainly reported based on evaluations of women who have been referred to hospitals and clinics to receive cervical cancer screening. Furthermore, the women examined in previous studies have come from a wide range of ages and, thus, information on the rates of HPV infection in young women, particularly those in their twenties, is lacking. For instance, Inoue *et al.*[27] reported on a large scale study evaluating HPV testing in over 8000 women in the Ishikawa Prefecture. The median age of these women was 36 years but the age span of the participants extended from 14 to 83 years of age. A further concern is that

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Clinical trial registry: Clinicaltrials.gov.
Name: Human papillomavirus vaccine (Cervarix) efficacy, immunogenicity and safety trial in adult Japanese women with GSK Biologicals HPV-16/18 vaccine.
Number: NCT00316693.

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these studies were usually conducted in small geographic areas relating to a particular hospital, clinic or prefecture. Very little information regarding nationwide HPV prevalence is available.

We have recently reported the results of a clinical study to evaluate the bivalent HPV-16/18 AS04-adjuvanted vaccine (Cervarix®, GlaxoSmithKline Biologicals) in healthy Japanese women aged 20–25 years.[28–30] While the results of this study showed that the HPV-16/18 AS04-adjuvanted vaccine was effective and immunogenic, with a clinically acceptable safety profile in the population studied, an accurate knowledge of the actual HPV infection rates in young healthy Japanese women could further confirm the importance of vaccination against HPV types 16 and 18. We therefore determined the HPV infection status of each woman at study entry as an indicator of the national infection rates. In particular, we analyzed DNA of 25 HPV types (14 oncogenic: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68; 11 non-oncogenic types: 6, 11, 34, 40, 42, 43, 44, 53, 54, 70 and 74) using PCR and report here the baseline prevalence of both the oncogenic and non-oncogenic HPV types in the cervical cytology samples of young healthy Japanese women.

Materials and Methods

Healthy Japanese women aged 20–25 years were recruited in this phase II double-blind (observer-blind) controlled, randomized, multicenter study (104798, NCT00316693) between April and October 2006. The 13 centers were located in Aomori, Tokyo, Fukui, Osaka, Hiroshima, Miyazaki and Kagoshima in Japan. Study participants were not screened before enrolment with respect to baseline serological, cytological or HPV DNA status. Inclusion and exclusion criteria were as previously described.[30] The study was conducted following the Declaration of Helsinki (version 1996) and all participants provided written informed consent. All recruitment materials, informed consent, protocol, and amendments were approved by independent institutional review boards.

Subjects were randomized in a 1:1 fashion to receive either the HPV-16/18 AS04-adjuvanted vaccine (containing 20 μg of HPV-16 L1 virus-like particle (VLP) and 20 μg of HPV-18 L1 VLP adjuvanted with 50 μg 3-*O*-desacyl-4′-monophosphoryl lipid A and 0.5 mg aluminium hydroxide) or a hepatitis A vaccine licensed in Japan (Aimmugen®, Chem-Sero-Therapeutic Research Institute, Kumamoto, Japan; containing 0.5 μg of inactivated hepatitis A antigen) as the control vaccine. Both vaccines were administered intramuscularly according to a 0-, 1-, and 6-month schedule. Investigators obtained cervical specimens with a cervical brush for cytology and HPV DNA as previously described.[18,28–31]

HPV DNA isolated from the cytology specimen was amplified from an aliquot of purified total DNA with the SPF_{10} broad-spectrum primers. These primers amplify a 65 bp region of the L1 gene and the generic amplification products were detected by hybridization on a microtiter plate (DEIA). HPV-positive specimens were typed by reverse hybridization LiPA. The broad-spectrum PCR SPF_{10} HPV $LiPA_{25}$ version 1 and SPF_{10} HPV DEIA (manufactured by Labo Biomedical Products, Rijswijk, the Netherlands based on licensed INNOGENETICS SPF_{10} technology) detected 25 HPV types: 14 oncogenic (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and 11 non-oncogenic HPV types (6, 11, 34, 40, 42, 43, 44, 53, 54, 70 and 74). If a sample was negative for HPV-16 or HPV-18 by the SPF_{10}-$LiPA_{25}$ system, type-specific PCR was performed to confirm the absence of these types using HPV-16 primers that amplified a 92 bp segment of the E6/E7 gene and HPV-18 primers that amplified a 126 bp segment of the L1 gene.[18,28–31]

Cytology was assessed by liquid-based cytology (ThinPrep, Cytyc Corporation, Marlborough, MA, USA) using a central laboratory (Quest Diagnostics, Teterboro, NJ, USA). Cytology results were reported using the 2001 Bethesda system and cytological abnormalities included: (i) ASC-US; (ii) LSIL; (iii) ASC-H; (iv) HSIL; and (v) AGC. Protocol guidelines recommended colposcopy after one report of HSIL or ASC-H. Biopsy was required for any suspected lesions on colposcopy. The central laboratory (Quest Diagnostics) processed and interpreted results from histology samples. All CIN endpoints were confirmed by an expert histopathology review panel that was blinded to vaccine status, HPV DNA status before biopsy, and cytology reports.

The enrolment target of 1000 unscreened women was estimated to provide 800 women who were DNA negative for HPV-16 or HPV-18 at month 0 and 6, and who would be evaluable for assessment of the primary endpoint in the according to protocol group for efficacy analysis. All analyses were based on the TVC; however, some values were absent due to missing or non-evaluable samples. Data is presented as percentage of subjects per group along with the actual number of subjects. Statistical analysis is descriptive in nature.

Results

One thousand and forty healthy Japanese women aged 20–25 years old (mean age: 22.5) were enrolled in 13 study sites in Japan. All women were vaccinated and included in the TVC: 519 women in the HPV group (HPV-16/18 AS04-adjuvanted vaccine) and 521 women in the control group (HAV group, hepatitis A vaccine licensed in Japan). The study sites were located in seven areas with the following recruitment numbers: Tokyo 457 (44.0%), Aomori 160 (15.4%), Osaka 136 (13.1%), Fukui 128 (12.3%), Kagoshima 91 (8.8%), Hiroshima 39 (3.8%) and Miyazaki 29 (2.8%).

The distributions of the tested HPV types in the TVC (irrespective of cytology) and for each cytological status are shown in Table 1. Data was not available for one participant due to a missing sample. Three hundred and fifty-five women (34.2%) of the TVC (irrespective of cytology) tested positive for HPV DNA at study entry. In particular, oncogenic and non-oncogenic HPV types were detected in 304 (29.3%) and 112 (10.8%) women. HPV-52 (8.1%, 84/1039) was the most frequently detected HPV type, followed by HPV-16 (6.5%, 68/1039), HPV-51 (4.5%, 47/1039), HPV-18 (4.0%, 42/1039) and HPV-31 (3.8%, 39/1039).

The majority of women (87.4%, 908/1039 women) had no cytological abnormalities; 12.6% (131/1039) had cytological abnormalities including ASC-US (4.5%, 47/1039), LSIL (7.2%, 75/1039), HSIL (0.7%, 7/1039) and ASC-H (0.2%, 2/1039). Overall, 26.1% of women with normal cytology (237/908) were positive for HPV DNA (Table 1). In contrast, of the 131 women who had abnormal cytology, 90.1% (118/131) were positive for HPV DNA. The HPV positivity rate was also high for women diagnosed with ASC-US (83.0%, 39/47), LSIL (93.3%, 70/75) and 100% (9/9) for women diagnosed to have HSIL or ASC-H. HPV-16 (23.7%, 31/131) was the most frequently detected HPV type, followed by HPV-52 (19.8%, 26/131), HPV-31 and HPV-56 equally detected in 13.0% (17/131) and HPV-51 (12.2%, 16/131). Fifty-five percent (5/9) of the women diagnosed as HSIL or ASC-H had HPV-16 detected in their cytological specimens. The nine women with HSIL or ASC-H had a colposcopy after the cytology testing. CIN3 was diagnosed in five women, CIN2 in one woman, CIN1 in one woman and two women had no lesions. Four of the five women who were diagnosed with CIN3 and the woman diagnosed with CIN2 were positive for HPV-16. The positivity rate of HPV-16 in the women diagnosed as CIN2 or CIN3 reached 83.3% (5/6).

The number of multiple HPV infections is illustrated by Table 2. Multiple infections were observed in 140 women (13.5%, 140/1039) of the TVC (irrespective of cytology),

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Table 1. HPV DNA genotype status distribution in cervical samples, and cytological and histological status at study entry (total vaccinated cohort)

HPV types	Total (n = 1039)†		Cytological status												Histological status‡	
			Normal (n = 908, 87.4%)		Abnormal (n = 131, 12.6%)		ASC-US (n = 47, 4.5%)		LSIL (n = 75, 7.2%)		HSIL (n = 7, 0.7%)		ASC-H (n = 2, 0.2%)		CIN2+ (n = 6, 0.6%)	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Oncogenic HPV																
16	68	6.5	37	4.1	31	23.7	11	23.4	15	20.0	4	57.1	1	50.0	5	83.3
18	42	4.0	27	3.0	15	11.5	3	6.4	12	16.0	0	0	0	0	0	0
31	39	3.8	22	2.4	17	13.0	4	8.5	11	14.7	2	28.6	0	0	1	16.7
33	1	0.1	0	0	1	0.8	1	2.1	0	0	0	0	0	0	0	0
35	8	0.8	6	0.7	2	1.5	0	0	1	1.3	1	14.3	0	0	1	16.7
39	37	3.6	24	2.6	13	9.9	4	8.5	9	12.0	0	0	0	0	0	0
45	4	0.4	1	0.1	3	2.3	1	2.1	2	2.7	0	0	0	0	0	0
51	47	4.5	31	3.4	16	12.2	5	10.6	10	13.3	0	0	1	50.0	0	0
52	84	8.1	58	6.4	26	19.8	11	23.4	12	16.0	3	42.9	0	0	0	0
56	37	3.6	20	2.2	17	13.0	6	12.8	11	14.7	0	0	0	0	0	0
58	31	3.0	17	1.9	14	10.7	4	8.5	9	12.0	1	14.3	0	0	1	16.7
59	7	0.7	3	0.3	4	3.1	1	2.1	3	4.0	0	0	0	0	0	0
66	35	3.4	23	2.5	12	9.2	1	2.1	11	14.7	0	0	0	0	0	0
68	17	1.6	12	1.3	5	3.8	2	4.3	2	2.7	1	14.3	0	0	0	0
Total	304	29.3	197	21.7	107	81.7	36	76.6	62	82.7	7	100	2	100	6	100
Non-oncogenic HPV																
6	25	2.4	17	1.9	8	6.1	2	4.3	6	8.0	0	0	0	0	0	0
11	6	0.6	5	0.6	1	0.8	0	0	1	1.3	0	0	0	0	0	0
34	12	1.2	6	0.7	6	4.6	4	8.5	2	2.7	0	0	0	0	0	0
40	9	0.9	6	0.7	3	2.3	1	2.1	2	2.7	0	0	0	0	0	0
42	4	0.4	3	0.3	1	0.8	0	0	1	1.3	0	0	0	0	0	0
43	12	1.2	7	0.8	5	3.8	2	4.3	3	4.0	0	0	0	0	0	0
44	3	0.3	3	0.3	0	0	0	0	0	0	0	0	0	0	0	0
53	38	3.7	29	3.2	9	6.9	2	4.3	7	9.3	0	0	0	0	0	0
54	14	1.3	14	1.5	0	0	0	0	0	0	0	0	0	0	0	0
70	2	0.2	2	0.2	0	0	0	0	0	0	0	0	0	0	0	0
74	3	0.3	2	0.2	1	0.8	1	2.1	0	0	0	0	0	0	0	0
Total	112	10.8	82	9.0	30	22.9	9	19.1	21	28.0	0	0	0	0	0	0
Overall total	355	34.2	237	26.1	118	90.1	39	83.0	70	93.3	7	100	2	100	6	100

†Data was not available for one participant due to missing sample. ‡Nine subjects who were diagnosed HSIL or ASC-H at study entry had a colposcopy after the cytology testing. ASC-H, atypical squamous cells, cannot exclude HSIL; ASC-US, atypical squamous cell of undetermined significance; CIN, cervical intraepithelial neoplasia; HSIL, high-grade squamous intraepithelial lesion; HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesion.

Table 2. Number of human papillomavirus (HPV) infections at study entry in the total vaccinated cohort (TVC) and in HPV positive women (HPV+)

Number of infections	n	TVC	HPV+
		% (n = 1039)†	% (n = 355)
6 types	1	0.1	0.3
5 types	6	0.6	1.7
4 types	14	1.3	3.9
3 types	40	3.8	11.3
2 types	79	7.6	22.2
1 type	215	20.7	60.6

†Data was not available for one participant due to missing sample.

including one woman (0.1%) testing positive for six HPV types (45/51/52/53/56/58) and six women (0.6%) positive for five HPV types. Fourteen women (1.3%) were positive for four HPV-types, 40 women (3.8%) positive for three types and 79 of the women (7.6%) had two HPV types. When only the HPV positive women (n = 355) are examined, these percentages increase to 0.3% testing positive for six HPV types, 1.7% for five types, 3.9% for four types, 11.3% for three types and 22.2% of the HPV-positive women had two HPV types (Table 2).

Discussion

This study is the first to evaluate the HPV prevalence for healthy young Japanese women nationwide, based in seven different regions of Japan. Study sites were well-distributed with the most northern site (Aomori) located in the farthest north prefecture of the main island and the most southern site (Kagoshima) located in the farthest south prefecture of the third island of Japan. Healthy Japanese women aged 20–25 years of age were recruited for a clinical study to assess the efficacy, immunogenicity and safety of the HPV-16/18 AS04-adjuvanted vaccine against HPV. We assessed the baseline HPV status using a sensitive PCR and cytology of the women enrolled in this clinical study and these are the data we present.

Although the overall HPV prevalence was slightly higher in the present study, type-specific prevalence data in Japanese women with normal cervical cytology was comparable to two large scale meta-analyses of Japanese data.[32,33] HPV-52 was also the most prevalent HPV type with HPV-16 second in these findings. While HPV-51 was the third most prevalent HPV type based on the results of the current study and of Miura *et al.*,[33] HPV-51 was fourth in the study of Konno *et al.*[32] with HPV-58 third. Previous findings from other Japanese studies were strongly supported by the large dataset used for the meta-analysis, in particular for age groupings.[32,33] HPV prevalence in women in the Asia Pacific region who were in their twenties with normal cytology was highest in Australia (30.1%), followed by Japan (23.1%), India (13.2%), Korea (12.7%) and Taiwan (9.9%).[34] Our data for women aged 20–25 years with normal cytology is relatively high (26.1%) compared to other women from the Asia Pacific region, with the exception of Australian women, but similar to that previously reported for Japanese women.[34] Interestingly, the four HPV genotypes with the highest prevalence in this study matched four of the five most common high-risk HPV genotypes in Asian women with normal cytology from China, Singapore, Taiwan and Indonesia.[34]

While HPV-52 is the most prevalent in the Japanese general population, HPV-16 is more closely associated with high-grade precursor lesions and invasive cancer.[32,33] The meta-analysis of Japanese data indicated that in normal cytological samples the prevalence of HPV-16 was 0.84% and of HPV-18 was <0.50%.[32] However, these data were compiled based on screenings taken of women of all ages. While HPV prevalence in the USA was common among women between the ages of 14 and 59 years old, prevalence was highest in women between the ages of 20 and 24 years old.[35] This current nationwide study specifically examined young women aged 20–25 years and shows a higher prevalence of the HPV-16 and HPV-18 types, particularly HPV-18 in normal cytology. Furthermore, HPV prevalence in Japanese women with normal/negative cytology appears to decrease with aging; for example from 26.1% at 22.5 years (average age) in the present study to 22.5% at 35.0 years[26] and 10.2% at 52.4 years.[36] Although these studies were not based in the same geographic locations and used different primer systems for their PCR analysis, it has been previously observed that HPV-16 and HPV-18 DNA positivity appears to be very high in Japanese women aged 20–29 years with CIN2-3 or invasive cervical cancer.[26] Therefore, it is possible that, due to high prevalence of HPV-16 and HPV-18 in Japanese women with normal cytology, cervical cancer caused by these HPV types will increase in the future.

As described above, HPV type distribution in invasive cervical cancers is very similar among countries of eastern Asia (including Japan, China/Hong Kong/Taiwan and Korea). HPV-16 and -18 are the most common types accounting for approximately 70% with the next three most common types being HPV-58, HPV-52 and HPV-33 accounting for an additional 10%.[26,37] Overall HPV and oncogenic HPV prevalence were 34.1% and 29.2% in all study participants. In the pivotal clinical study (PATRICIA) to evaluate the HPV-16/18 AS04-adjuvanted vaccine with approximately 18 000 women aged 15–25 years conducted in Asia Pacific, Europe, and North and South America, oncogenic HPV types were detected in 20.1% of all study participants.[14] Particularly, the prevalence of HPV-16 and HPV-18 was 5.4% and 2.3% in the participants of the clinical study. The broader age range of the women in that study precludes direct comparison of the Japanese data presented here; however, the prevalence of oncogenic HPV types, especially HPV-16 (6.5%) and HPV-18 (4.0%), in Japanese women is higher than that in other populations.

Cervical cancer is preventable if precancerous lesions are detected early enough. Cytological analysis to detect abnormalities such as LSIL and HSIL is used in combination with histology to define the early stages (low-grade cervical lesions) and advanced stages (high-grade cervical lesions) of the disease.[38] The strong association between oncogenic HPV prevalence, particularly of HPV-16, and cytological abnormalities such as LSIL and HSIL observed in this study follows the high incidences of HPV in invasive cervical cancer, HSIL and LSIL throughout Asia.[37] Indeed, an increasing prevalence of oncogenic HPV with increasing cervical lesion severity has been previously reported in Japanese women,[26,32,33] particularly of the HPV-18 genotype.[26] In agreement with this observation, there was also a good correlation between the prevalence of oncogenic HPV types and HSIL across the different parts of Europe.[39]

While the majority of HPV-positive women in this study were infected with a single HPV-type (60.6%), multiple HPV infections were observed in the remaining 39.4%. This is much higher than the 12.0% previously reported for Japanese women with normal cytology and HPV positive that were also positive for multiple HPV infection types.[26] The higher rate observed in our study is most likely due to the differences in the ages of the groups examined as we specifically examined women aged 20–25 years of age. Women aged 20–24 years of age had the highest prevalence of HPV infection compared to younger (14–24 years) and older (25–29, 30–39, 40–49, or 50–59 years) women, especially when restricting the analysis to sexually active females, in a US study.[35] Furthermore, the rate of multiple infection has been demonstrated to be higher in younger than older women in a Danish study and was also linked to sexual behavior.[40] Therefore, HPV vaccines that provide protection against multiple HPV types may be more effective in preventing cervical cancer.

The introduction of systematic cytological screening has resulted in a fall of the death rate from cervical cancer in many countries.[41] Vaccination against HPV-16 and HPV-18 would theoretically decrease by approximately 40% the number of oncogenic HPV-positive findings in screening programs.[39] As HPV type distribution in invasive cervical cancers is very similar among countries of eastern Asia (including Japan, China/Hong Kong/Taiwan and Korea), approximately 70% of invasive cervical cancer cases could be potentially prevented by an HPV-16/18 prophylactic vaccine.[18] Moreover, this could increase to approximately 80% if the vaccine targeted additional HPV types, such as HPV-31 and HPV-45, as well as HPV-16 and HPV-18.[37] Both HPV-16 and HPV-18 are phylogenetically related to other HPV subtypes (HPV-16: HPV-31, -33, -35, -52, -58, -67; HPV-18: HPV-39, -45, -59, -68, -70),[42] suggesting that vaccines targeting HPV-16/18 could cross-react with these subtypes. Cross-protection data have been reported for both the bivalent and the quadrivalent vaccines.[11,16,43,44] The bivalent vaccine demonstrated cross-protection against persistent infection with HPV-31, -33 and -45 of 78.7%, 45.7% and 75.7%[16] while the quadrivalent showed cross protection of approximately 46% against persistent infection with HPV-31.[44] Vaccination against HPV may, thus, provide extra protection against cervical cancer caused by non-vaccine HPV types, especially those vulnerable to infection by multiple HPV types.

Indeed, the increasing prevalence of HPV-16 and HPV-18 observed in young healthy Japanese women in this study may indicate that the incidence of cervical cancer in Japan caused by HPV-16/18 will increase in the future. Therefore, a greater emphasis needs to be placed on providing information on HPV and screening for oncogenic types of HPV, in particular HPV-16 and HPV-18. Safe and effective vaccination against HPV-16 and HPV-18 will help prevent the increase of HPV infection and subsequent development of cervical cancer. Furthermore, the

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HPV prevalence data reported here in healthy young Japanese women would be very useful for monitoring the changes in HPV prevalence after widespread HPV vaccination in Japanese women in the future.

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Disclosure Statement

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Abbreviations

AGC	atypical glandular cells
ASC-H	atypical squamous cells, cannot exclude HSIL
ASC-US	atypical squamous cell of undetermined significance
CIN	cervical intraepithelial neoplasia
DEIA	DNA enzyme immunoassay
HPV	human papillomavirus
HSIL	high-grade squamous intraepithelial lesion
LiPA	line probe assay
LSIL	low-grade squamous intraepithelial lesion
PCR	polymerase chain reaction
TVC	total vaccinated cohort
VLP	virus-like particle

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Identification of Human Papillomavirus Type 58 Lineages and the Distribution Worldwide

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Background. Human papillomavirus type 58 (HPV-58) accounts for a much higher proportion of cervical cancers in East Asia than other types. A classification system of HPV-58, which is essential for molecular epidemiological study, is lacking.

Methods and results. This study analyzed the sequences of 401 isolates collected from 15 countries and cities. The 268 unique concatenated E6-E7-E2-E5-L1-LCR sequences that comprised 57% of the whole HPV-58 genome showed 4 distinct clusters. L1 and LCR produced tree topologies that best resembled the concatenated sequences and thus are the most appropriate surrogate regions for lineage classification. Moreover, short fragments from L1 (nucleotides 6014–6539) and LCR (nucleotides 7257–7429 and 7540–52) were found to contain sequence signatures informative for lineage identification. Lineage A was the most prevalent lineage across all regions. Lineage C was more frequent in Africa than elsewhere, whereas lineage D was more prevalent in Africa than in Asia. Among lineage A variants, sublineage A2 dominated in Africa, the Americas, and Europe, but not in Asia. Sublineage A1, which represents the prototype that originated from a patient with cancer, was rare worldwide except in Asia.

Conclusions. HPV-58 can be classified into 4 lineages that show some degree of ethnogeographic predilection in distribution. The evolutionary, epidemiological, and pathological characteristics of these lineages warrant further study.

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Human papillomavirus (HPV) infection is a necessary, although insufficient, cause of cervical cancer, which is still the second leading cancer to affect women worldwide [1]. Two prophylactic vaccines (Cervarix and Gardasil) targeting the 2 most prevalent high-risk HPV types (HPV types 16 and 18) found in cervical cancer are available [2, 3]. These vaccines are expected to prevent ~70% of cervical cancers worldwide [4]. There remain at least 13 high-risk HPV types not targeted by the

current vaccines that account for the remaining cervical cancers [5]. Globally, HPV types 31, 33, and 45 form the second group and HPV types 35, 52, and 58 form the third group in the ranking of cancer association [6]. However, the disease impact associated with these other HPV types shows considerable geographical variation. As shown in a meta-analysis, HPV type 58 (HPV-58) was found in 3.3% of cervical cancers globally and 5.6% of cervical cancers in Asia, whereas the prevalence in high-grade cervical intraepithelial lesions was 7.0% globally and 12.2% in Asia [4]. Studies from East Asian populations have reported an even higher rate. For instance, HPV-58 was detected in 26% of cervical squamous cell carcinoma in Shanghai [7], 16% in South Korea [8], 10% in Hong Kong [9] and Taiwan [10], and 8% in Japan [11]; and HPV-58 ranked third in cervical cancer cases from Asia overall [12]. Although HPV-58 may not play an etiological role in all these cases, as some of them are found in coinfections with other high-risk types, the disease impact conferred by HPV-58, especially in East Asian populations, cannot be neglected. The reason for a geographical or ethnical predilection of HPV-58-associated cervical neoplasia is not fully understood. Previous studies have suggested that host genetic factors and the circulation of variants with higher oncogenicity could play a role [13, 14]. To date, information on sequence variability of HPV-58 is very limited. This study was conducted to elucidate the phylogenetic relationship between HPV-58 variants collected worldwide to establish a classification system that will facilitate further study on the oncogenic potential of HPV-58.

MATERIALS AND METHODS

Study Samples

Cervical, vaginal, or anal samples were collected by study collaborators. Those samples that had tested positive for HPV-58 were transferred to a central laboratory for sequence analysis. The quality of DNA was assessed by amplifying a 1039-bp fragment of the L1 region, and the identity of HPV-58 was ascertained by demonstrating a nucleotide sequence similarity of >90% compared with the corresponding L1 fragment of the prototype (GenBank accession no. NC_001443). Altogether, 401 samples collected from 15 geographical locations had sufficient DNA quality for sequencing of the whole length of the E6, E7, E2 (containing E4), E5, L1, and LCR regions (Table 1). All samples except 37 anal swab specimens from men in the United States were cervical, vaginal scrape, swab, or tissue specimens from women. The distribution of cervical pathology status is shown in Table 1.

Nucleotide Sequencing

The whole lengths of E6, E7, E2 (containing E4), E5, L1, and LCR were amplified separately by polymerase chain reaction (PCR) using primers designed on the basis of the prototype (http://www.ncbi.nlm.nih.gov/genbank accession no. NC_001443; primer sequences are shown in the Supplemental Data). Briefly, the PCR was conducted in a 50-μL reaction mix containing 4 μL of extracted DNA, 200 μmol/L deoxynucleotide triphosphates, forward and reverse primers at .25 μmol/L each, and 1.25 U of HotStarTaq Plus polymerase (Qiagen). The cycling conditions were as follows: activation of polymerase at 95°C for 5 min, 40 cycles of denaturation at 94°C for 50 s, annealing at 56°C–60°C for 50 s, and extension at 72°C for 50–70 s, followed by a final extension at 72°C for 8 min. Amplification was visualized by agarose gel electrophoresis. Samples with insufficient amplification product for sequencing were subjected to another round of PCR using the nested primers.

PCR products were purified by Microspin S-400 columns (GE Healthcare). Ten microliters of the purified PCR products were mixed with 2 μL of BigDye Terminator sequencing reaction mix (version 3.1; Applied Biosystems), 3 μL of 5× sequencing buffer, and 3.2 pmol of the sequencing primer; and made up to a final volume of 20 μL according to the manufacturer's instructions. The cycling conditions for the labeling PCR were 25 cycles at 95°C for 15 s, 50°C for 15 s, and 65°C for 75 s. Fluorescence-labeled PCR products were purified with DyeEx (Qiagen) and run on an ABI 3130 automated sequence analyzer (Applied Biosystems). Sequence data were obtained from both directions and analyzed with SeqScape software (version 2.5; Applied Biosystems). Mutations that occurred only once were confirmed by repeating from the original sample.

Naming of Variants and Phylogenetic Tree Construction

Variant sequences were named WW for "worldwide," followed by a number according to its prevalence as found in this study. The concatenated nucleotide sequences from the 5 complete open reading frames (ORFs; E6, E7, E2, E5, and L1) and the LCR region of individual variants were used for phylogenetic tree construction. The tree construction processes were repeated for each genomic region to identify the most informative surrogate region for lineage classification of HPV-58 variants.

Maximum-likelihood trees were constructed using the program PAUP* (version 4.0b10) [15]. Modeltest (version 3.7) [16] was used to identify the best evolutionary model. A neighbor-joining tree was constructed as a starting tree, followed by a maximum-likelihood tree using the subtree pruning and regrafting (SPR) search approach. The data were bootstrap resampled 1,000 times. To verify the tree topologies observed from maximum-likelihood trees, the program MrBayes (version 3.1.2) [17] was used for Bayesian tree construction, with the nucleotide substitution model set according to the Modeltest results. The Markov chain Monte Carlo analysis was run for 5,000,000 generations with trees sampled at every 1,000 generations. A burn-in rate of 25% was used in summarizing the data. The trees were displayed with Figtree (version 1.1.2; http://tree.bio.ed.ac.uk/software).

Table 1. Distribution of Study Samples According to the Source of Collection and Cervical Pathology Status

Region, country or city	Total no. of specimens	No. of specimens of each cervical pathology status					
		Normal	ASCUS	LGSIL	HGSIL	Carcinoma	Unknown
Africa	...	...	...	...	...	...	...
Zimbabwe[a]	69	0	0	0	0	0	69
Americas	63	6	1	6	10	2	1[b]
Canada	10	2	0	4	4	0	0
United States	37	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]
Mexico	2	0	0	0	1	1	0
Argentina	6	3	1	0	0	1	1
Brazil	3	1	0	1	1	0	0
Honduras	5	0	0	1	4	0	0
Asia	238	57	23	57	66	31	4
Mainland China	3	0	0	0	0	0	3
Hong Kong	90	17	0	15	41	17	0
Taiwan	6	0	0	0	0	6	0
South Korea	119	30	23	40	22	4	0
Japan	14	9	0	2	1	1	1
Thailand	6	1	0	0	2	3	0
Europe	31	16	1	9	3	0	2
United Kingdom	14	6	0	6	2	0	0
Italy	17	10	1	3	1	0	2
Total	401	83	26	68	72	34	81[b]

NOTE. ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; HGSIL, high-grade squamous intraepithelial lesions (including CIN2, CIN3, and severe dysplasia); LGSIL, low-grade squamous intraepithelial lesions (including CIN1 and mild dysplasia);

[a] Cervical or vaginal swabs.

[b] Not including 37 anal swab samples.

[c] Anal swab samples.

Geographical Distribution of Variant Lineages

The rate of detection of each variant lineage was compared among the 4 regions by use of a Pearson χ^2 test. When a significant difference was obtained between regions (defined as $P < .05$), detection rates between regions were compared 2 by 2 with a Fisher exact test, for a total of 6 comparisons. The level of significance for 6 possible comparisons was then set at .008 according to the Bonferroni correction.

The distribution of variant lineages among anal swabs that were collected from men at a single center in the United States was compared with that among cervical samples collected from other parts of the Americas by use of a Pearson χ^2 test or a Fisher exact test as appropriate. The association between variant lineage and cervical pathology status was assessed by a Fisher exact test. Two-tailed P values of $<.05$ were regarded as significant.

RESULTS

Lineage Classification

Altogether, 268 unique concatenated E6-E7-E2-E5-L1-LCR nucleotide sequences of HPV-58 variants were assembled. The lengths ranged from 4416 bp to 4462 bp, accounting for ~57% of the whole viral genome. Since none of the assembled sequences was identical to the prototype, the sequence available at GenBank (GenBank accession no. NC_001443) was used to assemble a concatenated prototype sequence to serve as a reference. The maximum-likelihood tree revealed 4 clusters (Figure 1), and the topology was same as that observed from the Bayesian tree.

Among all the genomic regions examined, L1 and LCR displayed tree topologies that most closely resembled that of the concatenated E6-E7-E2-E5-L1-LCR sequences. The L1 and LCR regions were considered to be most informative surrogate regions for phylogenetic grouping of HPV-58 variants when the full genome sequence is not available. The lineage containing the prototype was assigned as lineage A, and the others were arbitrarily designated as lineages B, C, and D. Figures 2A and 2B show the maximum-likelihood trees of the L1 and LCR sequences, which revealed topologies that were same as those observed from the Bayesian trees. The 2 most prevalent L1 variants, L1_WW001 and L1_WW002, were found in 26.7% and 11.7% of samples, respectively; whereas the L1_WW054 variant, found in .2% of samples, is the prototype. The 2 most prevalent LCR variants, LCR_WW001 and LCR_WW002, were found in 20.8% and 11.5% of isolates, respectively. The variant LCR_WW009, which was found in 3.6% of isolates, is

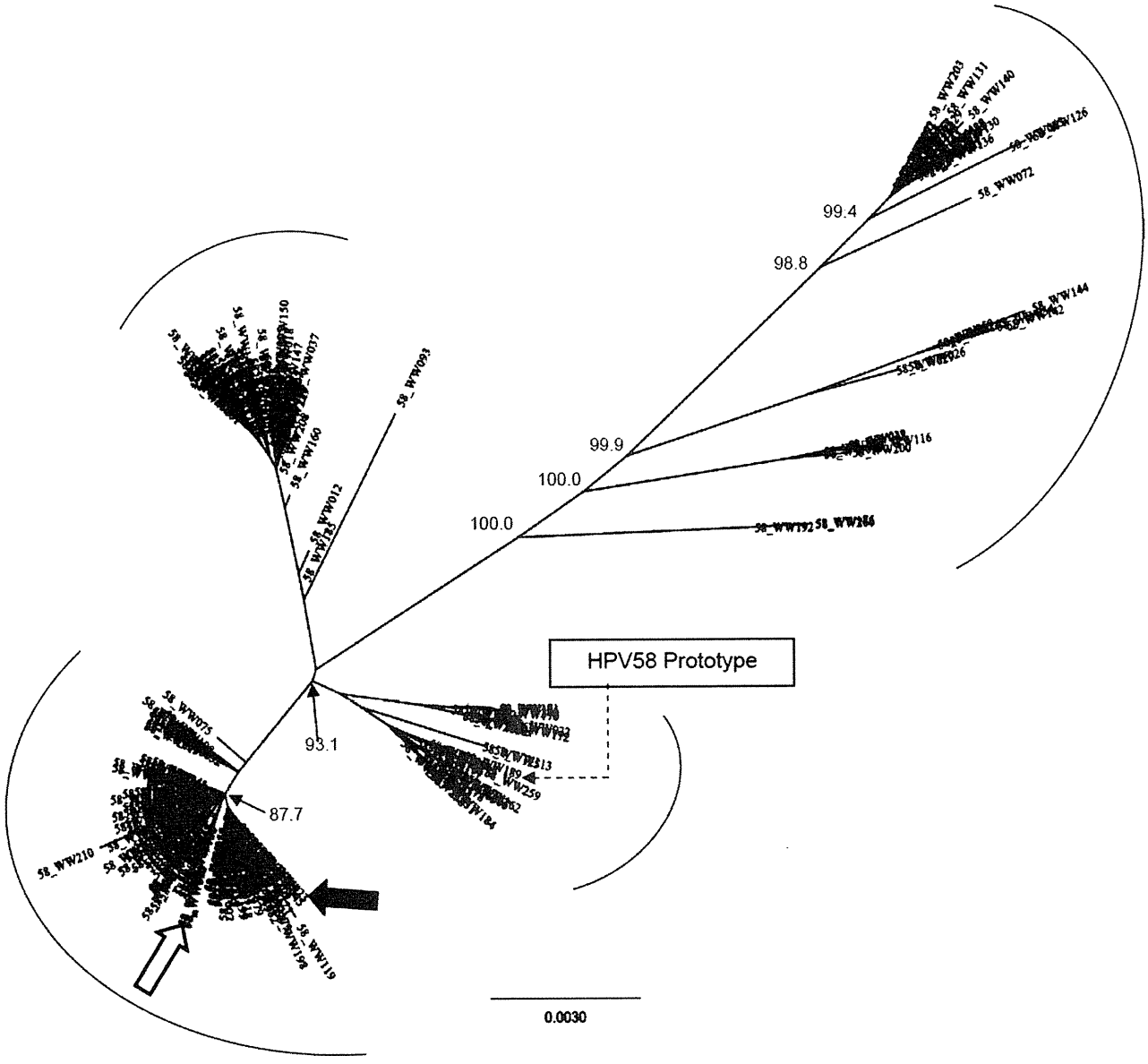


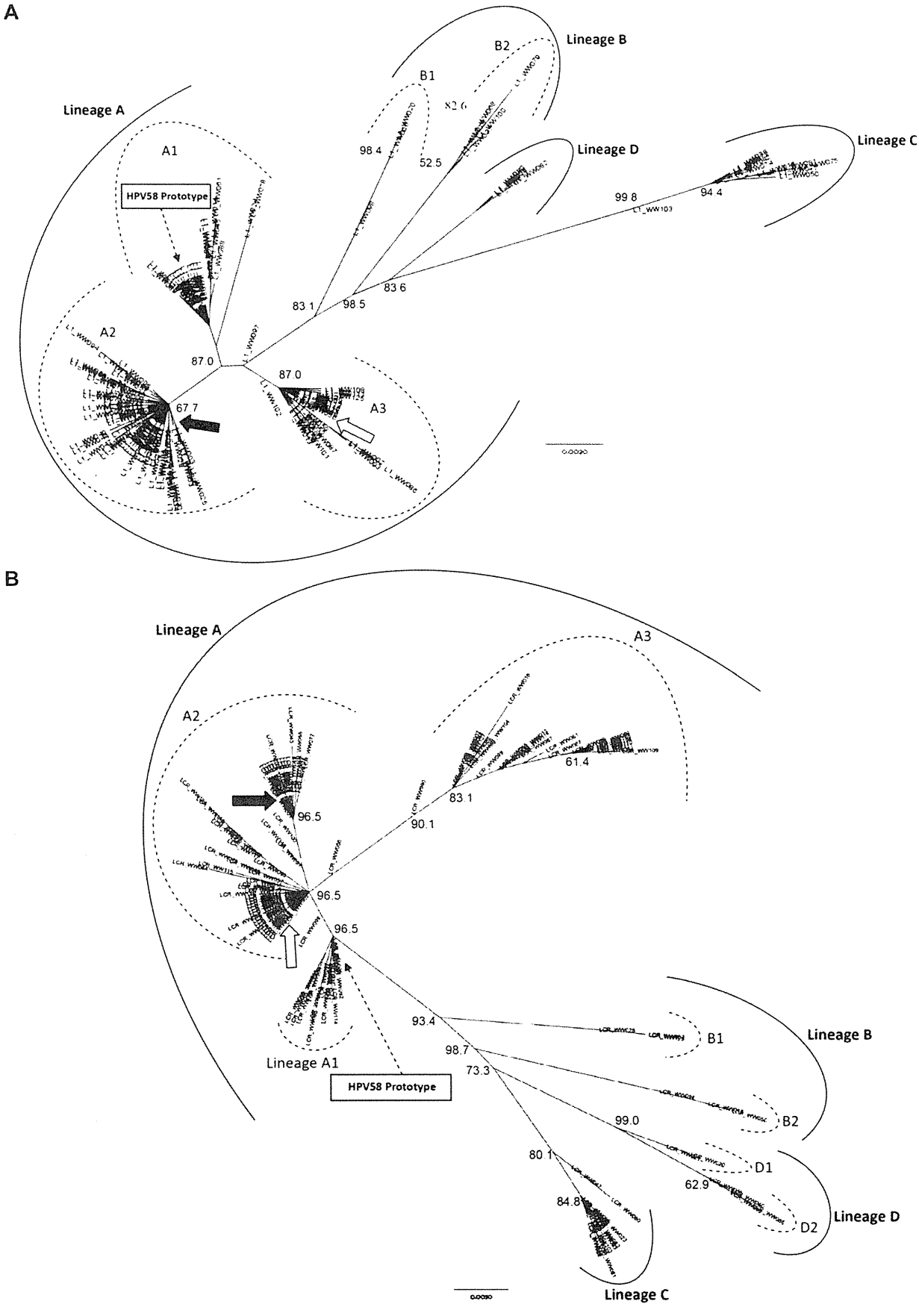
Figure 1. Phylogenetic tree constructed from concatenated E6-E7-E2-E5-L1-LCR nucleotide sequences of 268 human papillomavirus type 58 (HPV-58) Variants. The maximum-likelihood tree was constructed with the PAUP* program (version 4.0) using the GTR+I+G model for nucleotide substitution. Bootstrap values of >70% generated by 1,000 resamplings are shown. The length of the scale bar represents .003 substitutions per nucleotide position. The position of the HPV-58 prototype (http://www.ncbi.nlm.nih.gov/genbank accession no. NC_001443) is indicated. The 2 most prevalent variant sequences, WW_001 and WW_002, are marked with a black arrow and a white arrow, respectively.

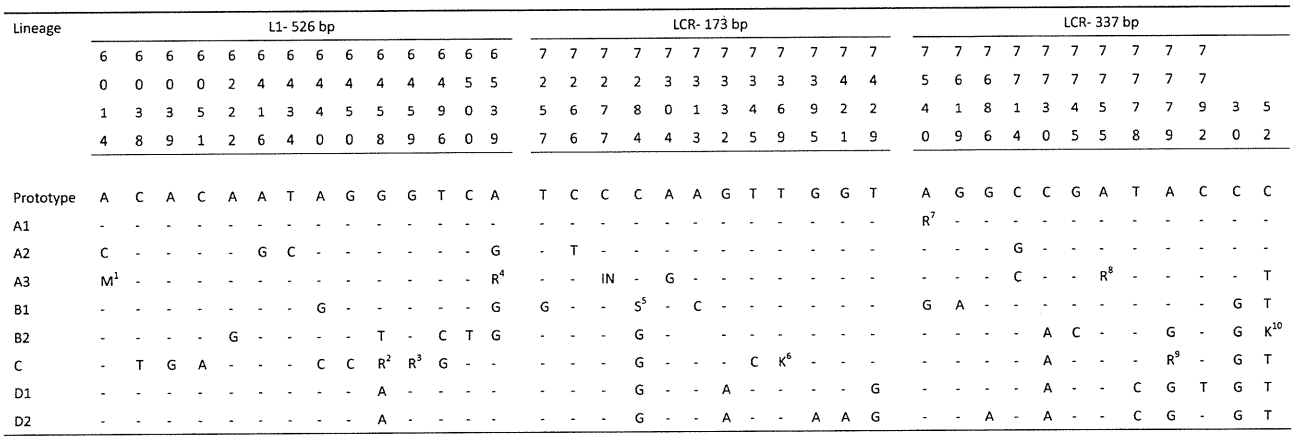
the prototype. None of the samples studied were found to harbor a mixture of variants.

The sequence variations of the L1 and LCR regions among different lineages were examined to identify shortest fragment or fragments that contain sequence signatures unique for each lineage. As a result, 3 fragments were found to be the best surrogate targets for lineage identification, including a 526-bp L1 fragment that corresponds to nucleotide position 6014–6539, a 173-bp LCR fragment at nucleotides 7257–7429, and another 337-bp LCR fragment at nucleotides 7540–52. The sequence variations at key positions are shown in Figure 3.

Geographical Distribution of HPV-58 Lineages

Figure 4 shows the distribution of HPV-58 lineages in each geographical location. Lineage A was the most prevalent lineage found worldwide (86.0% of isolates), as well as in each region (49.3%–95.8% of isolates). The prevalence of lineage A in Africa (49.3% of isolates) was significantly lower than in other regions (85.7%–95.8% of isolates; $P < .001$ for each comparison), whereas lineage A was significantly more frequent in Asia than in the Americas ($P = .007$). Lineage B was found in 2.5% of the isolates collected worldwide, ranging from none in Europe to 3.2% in the Americas. The number of lineage B isolates was too





Lineage	L1- 526 bp														LCR- 173 bp												LCR- 337 bp										
	6	6	6	6	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7		
	0	0	0	0	2	4	4	4	4	4	4	4	5	5	2	2	2	2	3	3	3	3	3	3	4	4	5	6	6	7	7	7	7	7	7	7	
	1	3	3	5	2	1	3	4	5	5	5	9	0	3	5	6	7	8	0	1	3	4	6	9	2	2	4	1	8	1	3	4	5	7	7	9	3	5
	4	8	9	1	2	6	4	0	0	8	9	6	0	9	7	6	7	4	4	3	2	5	9	5	1	9	0	9	6	4	0	5	5	8	9	2	0	2
Prototype	A	C	A	C	A	A	T	A	G	G	G	T	C	A	T	C	C	C	A	A	G	T	T	G	G	T	A	G	G	C	C	G	A	T	A	C	C	C
A1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R^7	-	-	-	-	-	-	-	-	-	-	-
A2	C	-	-	-	-	G	C	-	-	-	-	-	-	G	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-
A3	M^1	-	-	-	-	-	-	-	-	-	-	-	-	R^4	-	-	IN	-	G	-	-	-	-	-	-	-	-	-	-	C	-	-	R^8	-	-	-	-	T
B1	-	-	-	-	-	-	-	G	-	-	-	-	-	G	G	-	-	S^5	-	C	-	-	-	-	-	-	G	A	-	-	-	-	-	-	-	-	G	T
B2	-	-	-	-	G	-	-	-	-	T	-	C	T	G	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	-	G	-	G	K^{10}
C	-	T	G	A	-	-	-	C	C	R^2	R^3	G	-	-	-	-	-	G	-	-	-	C	K^6	-	-	-	-	-	-	-	A	-	-	-	R^9	-	G	T
D1	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	G	-	-	A	-	-	-	-	G	-	-	-	-	A	-	-	C	G	T	G	T
D2	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	G	-	-	A	-	-	A	A	G	-	-	A	-	A	-	-	C	G	-	G	T

Figure 3. Signature sequences for human papillomavirus type 58 (HPV-58) lineage identification. Numbers refer to nucleotide position of the HPV-58 prototype (http://www.ncbi.nlm.nih.gov/genbank accession no. NC_001443). A dash represents the same nucleotide as in the prototype. IN, insertion of 12 bp. For M^1, 91% of isolates have C and 9% have A; for R^2, 54.5% have G and 45.5% have A; for R^3, 91% have A and 9% have G; for R^4, 96% have G and 4% A; for S^5, 67% have C and 33% have G; for K^6, 86% have G and 14% have T; for R^7, 78.6% have A and 21.4% have G; for R^8, 96% have G and 4% have A; for R^9, 86% have G and 14% have A; and for K^{10}, 67% have T and 33% have G.

few for statistical analysis. Lineage C was found in 9.2% of isolates collected worldwide and was found significantly more frequently in Africa than in Asia, the Americas, or Europe ($P \leq .001$ for each comparison). Lineage D has a worldwide prevalence of 2.2% and seemed to be more frequent in Africa (8.7%). However, the number of lineage D isolates was too few for statistical analysis.

Since lineage A was the most prevalent lineage identified, a subgroup analysis was performed for the distribution of sublineages A1, A2, and A3. Sublineage A2 was the most frequently detected sublineage, accounting for 62.3% of lineage A isolates collected worldwide and dominating in Africa (94.1%), the Americas (87.0%), and Europe (93.1%). In contrast, sublineages A1, A2, and A3 were more evenly distributed in Asia, accounting for 16.2%, 47.8%, and 36.0%, respectively, of lineage A isolates found in this region. As a result, the proportion of lineage A isolates belonging to sublineage A2 was significantly lower in Asia than in other regions ($P < .001$), whereas the proportion belonging to sublineage A3 was significantly higher in Asia than in other regions ($P < .001$).

Of the 37 anal swabs collected from men in the United States, 30 (81.1%) were lineage A, 1 (2.7%) was lineage B, 5 (16.7%) were lineage C, and 1 (.3%) was lineage D. The proportion of each lineage among these samples was not significantly different ($P = .06–.4$) from that of samples collected from women in the Americas.

Lineage Distribution and Cervical Pathology

In Asia, lineage A accounted for 94.8% of high-grade squamous intraepithelial lesion (HGSIL) and carcinoma samples and 96.4% of samples of normal cytology, low-grade squamous intraepithelial lesion (LGSIL), and atypical squamous cells of undetermined significance (ASCUS); no significant difference in the distribution of lineage A compared with non-A lineages was found ($P = .745$). Similarly, lineage A accounted for the majority of HGSIL/carcinoma samples in Hong Kong (94.8%) and South Korea (92.3%), and no significant association between lineage and lesion severity was found ($P = 1.0$ for Hong Kong; $P = .120$ for South Korea). The number of samples available from other regions was too few for a similar statistical analysis.

DISCUSSION

Analysis of intratypic sequence variation of HPV can provide important information for the design of diagnostic tools, development of vaccines, identification of molecular markers for epidemiological studies, elucidation of implications of sequence variation on biological and pathological properties, and

Figure 2. Phylogenetic trees of L1 and LCR sequences of human papillomavirus type 58 (HPV-58) variants. The maximum-likelihood tree was constructed with the PAUP* program (version 4.0) using the GTR+I+G model for nucleotide substitution. Bootstrap values of key positions generated with resampling 1,000 times are shown. The length of the scale bar represents the number of substitutions per nucleotide position. The position of the prototype (http://www.ncbi.nlm.nih.gov/genbank accession no. NC_001443) is indicated. The 2 most prevalent variant sequences, WW_001 and WW_002, are marked with black and white arrows, respectively. *A*, L1 open reading frame of 121 variants (HM639317–HM639717). *B*, LCR open reading frame of 123 variants (HQ338950–HQ339350).

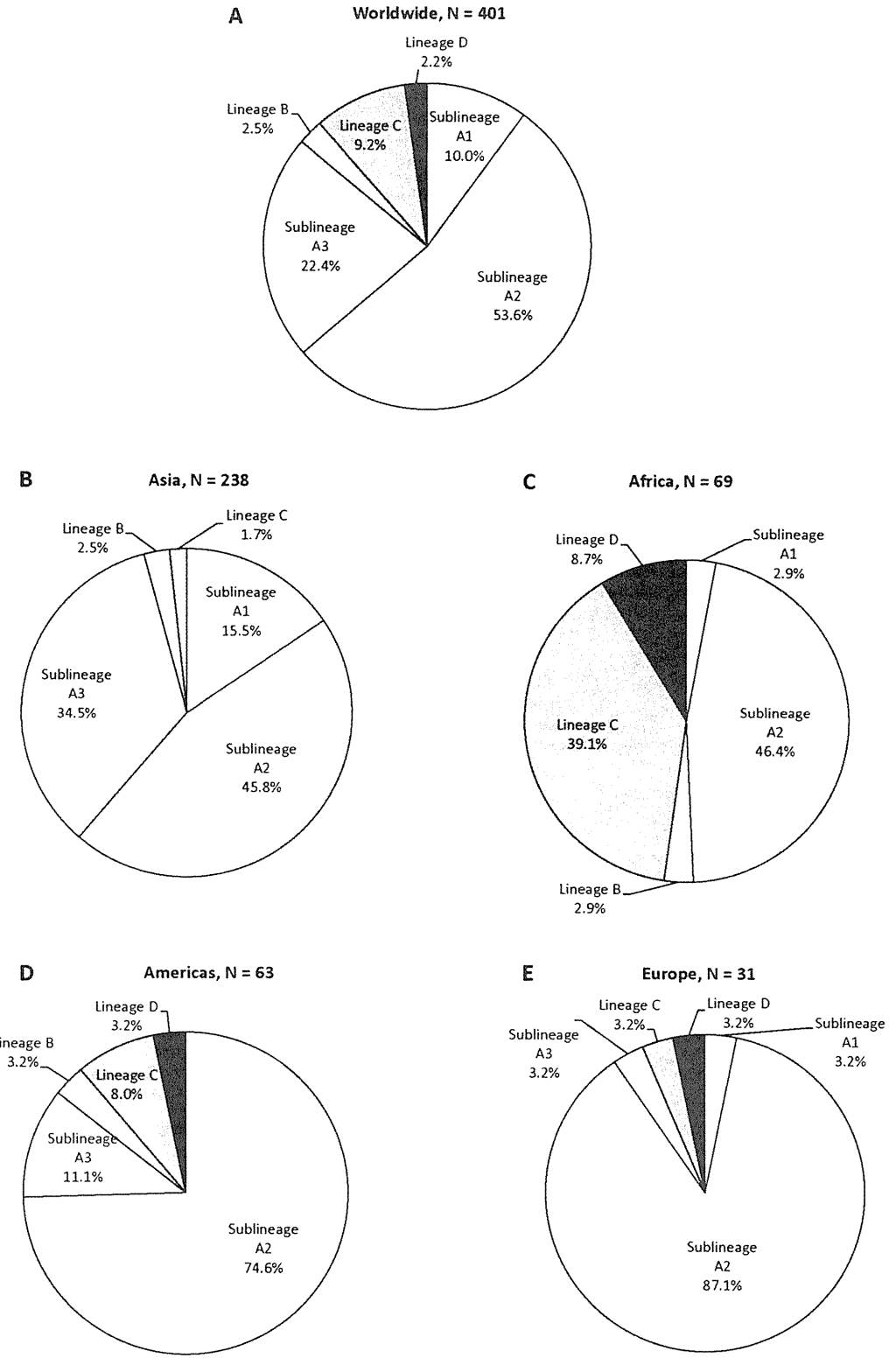


Figure 4. Geographical distribution of human papillomavirus type 58 (HPV-58) lineages. *A*, Worldwide ($N = 401$); *B*, Asia ($N = 238$); *C*, Africa ($N = 69$); *D*, the Americas ($N = 63$); *E*, Europe ($N = 31$).

understanding of the evolution and taxonomy of the virus [18, 19]. The currently available data are mainly derived from the 2 HPV types, HPV-16 and HPV-18, most commonly found in cervical cancers. Data on sequence variation of HPV-58 isolates collected worldwide are scarcely available [20, 21]. In this study, ~57% of the whole viral genome was sequenced. The selected

regions included L1, which is the most important region for defining HPV type and variant; LCR, which is the most variable region; E6, which contains informative signatures for HPV-16 variant lineage classification; E7, which has been reported to be more variable than E6 for HPV-58 [14]; and E2 and E5, which are important in oncogenesis. To our knowledge, the number of HPV-58 isolates examined in this study represents the largest reported collection sampled from multiple countries around the world. Nevertheless, one should be aware of the fact that the number of samples available from Africa and Europe for this study was relatively small, and thus the distribution of variants in these regions might not be fully elucidated.

Our analysis on the E6-E7-E2-E5-L1-LCR concatenated sequences of HPV-58 variants showed 4 phylogenetically distinct clusters, suggesting that HPV-58 variants had evolved into 4 lineages. We then attempted to identify genomic regions that could best reproduce the 4 clusters. Among the 7 genomic regions examined, 5 of them (E6, E7, E2, E4, and E5) were relatively conserved, as expected for these proteins. The tree topologies generated from these 5 regions were quite different from that of the concatenated sequences. On the other hand, the LCR and L1 regions displayed a tree topology that most closely resembled that of the concatenated sequences and were therefore regarded as the most informative surrogate regions for HPV-58 variant lineage classification. A similar topology was also observed by Calleja-Macias et al [20], who used a 461-bp fragment of LCR of 21 HPV-58 variants for tree construction.

The error frequency estimated for a standard Taq polymerase-based PCR ranges from 2×10^{-4} to 30×10^{-4} [21]. To minimize the chance of recording artificial sequence variations, we performed sequencing from both directions in independent PCRs. In addition, sequence variations observed only once were repeated. It is unlikely that the sequence variations presented are due to errors produced during the amplification process. The observed maximum nucleotide sequence divergence of the L1 ORF within each lineage ranged from .4% to 1.7%, and was 2.2% for all variants together. This limited sequence divergence indicates the absence of subtypes or intermediary genomes within the HPV-58 variants. This observation concurs with previous studies on other HPV types [20, 22]. HPV-58, as with other HPV types, probably has gone through genetic drifts that became amplified by founder effects and bottlenecks of evolution.

A clear association between phylogenetic clustering and the ethnogeographic origin of HPV-16 variants has been observed previously, and thus HPV-16 lineages were named as follows: E (European), As (Asian), AA (Asian American), and Af-1 and Af-2 (African 1 and 2) [23, 24]. The largest available series of analyses on HPV-58 variants was reported by Calleja-Macias et al [20], which included 101 samples from different parts of the world. Their analysis on a 461-bp fragment of LCR revealed 21 variants, showing a limited amount of diversification in unique geographical locations and no clear geographical association with any variants was observed. The present study allowed a more in-depth analysis based on a larger sample size. Although the ethnogeographic correlation for HPV-58 lineages was not as prominent as that for HPV-16, a predilection in distribution of HPV-58 lineages was observed in this study. Lineage A predominated in all regions except in Africa, where lineages A and C existed in comparable proportions. Although Asia comprised the largest number of samples in this study, none of them belonged to lineage D. The distribution of sublineages A1, A2, and A3 also displayed geographical variation. Although sublineage A2 predominated in Africa, the Americas and, Europe, a relatively higher frequency of sublineages A1 and A3 was found in Asia.

We hypothesize that lineage A (probably sublineage A2) was the oldest lineage, which disseminated with early human evolution and migration and had seeded into different parts of the world before other lineages emerged [24–25]. Host or environmental factors might have favored the emergence and spread of lineage C in Africa, whereas lineage D was difficult to establish in Asia.

We assigned A1 to the sublineage that contained the prototype, which was cloned from a patient with cervical cancer in Japan. In this study, sublineage A1 was rarely detected except in Asia. It is worthwhile to further investigate whether the reported higher contribution of HPV-58 to invasive cancers in East Asia is associated with a higher level of oncogenicity of sublineage A1 [7–12].

Since all the anal samples from men available for this study were collected from a single center in the United States, we compared their lineage distribution with samples from women collected from the rest of the Americas. The results showed that there were no significant differences between samples from men and those from women, and therefore pooling these samples together for the analysis of the geographical distribution of lineages was justified.

A potential limitation of the present study is the lack of sufficient samples to allow further analyses of the geographical distribution of variant lineages stratified according to cervical pathology status. Nevertheless, at least for Asia and the Americas, the proportion of samples with normal cytology or LGSIL was similar to that of samples with ASCUS, HGSIL, or carcinoma (48.7% and 48.0% of samples from Asia and the Americas, respectively, were normal/LGSIL), although Europe had a higher proportion of normal/LGSIL samples (86.2%), and information on cervical status for the samples from Africa was not known. We attempted to analyze the association between oncogenic risk and variant lineage on the basis of samples collected from Asia, Hong Kong, and South Korea, where a substantial number of samples in this study were collected, but no significant association was observed. However, such a result should not be regarded as final. Further studies are required to examine the oncogenic association of these variant lineages.

This study provides a detailed analysis on HPV-58 variant lineages and indicates that the distribution may be linked

ethnogeographically. Whether this reflects the survival fitness of these variants under different host genetic and environmental pressures or that some of these lineages are still slowly evolving and extending their ecological territories remains to be established. Further study on the evolution of HPV-58 and close monitoring of the possibility of type replacement by this virus following the widespread administration of HPV vaccines are warranted. It is worthwhile to further study the biological and pathological implications of this lineage classification system.

Supplementary Data

Supplementary tables are available online at http://jid.oxfordjournals.org.

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Short communication

Immunization coverage and natural infection rates of vaccine-preventable diseases among children by questionnaire survey in 2005 in Japan

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ABSTRACT

We performed questionnaire survey in 2005, just before the introduction of the MR vaccine, concerning child vaccination and/or infection history for measles, mumps, rubella, varicella, influenza, diphtheria–pertussis–tetanus (DPT), BCG, and Japanese encephalitis. The vaccination rate against measles and rubella did not exceed 95% at any age levels. As a result, children who had contracted measles and/or rubella were observed at all age levels. The vaccination rate was 95% or higher only for BCG and DPT. The vaccination rates for influenza, mumps, and varicella, although vaccination against which diseases was being performed voluntarily, were low, and outbreaks of these diseases were expected to persist. The vaccination rates at a low level for these infectious diseases might be one of the most possible risk factors to the high prevalence of the diseases in nursery schools (daycare centers), kindergartens, and elementary schools all over Japan.

For infectious disease control by preventive vaccination, information on the vaccination rates and prevalence of infections in local populations is indispensable for the design of approaches for susceptible populations, along with accompanying information on infection sources and routes. We performed a questionnaire survey in 2005 concerning child vaccination history and infection history with the parents of nursery school (daycare center), kindergarten, and elementary school children with the cooperation of persons involved in local health care, welfare, and educational services. Measles, rubella, epidemic parotitis (mumps), varicella, influenza, pertussis (DPT), BCG, and Japanese encephalitis were selected as diseases (vaccines) of interest. The questionnaire consisted of questions concerning the children's histories of vaccination and infections (e.g., Did your child receive the vaccine within the last 1 year, or earlier? Did your child contract the disease within the last 1 year, or earlier?). These questions were designed to be answered by the children's parents, by selecting the correct responses from among the suggested answers and checking them off (√). In addition, attention was given to the distinguishing whether the vaccinations were performed routinely (during the period stipulated by the law) or voluntarily, and to collecting the data without individual identification. A document explaining the objective of the questionnaire survey and a letter of request for cooperation were submitted to the welfare sections, health promotion sections (preventive vaccination centers), child rearing support (child care) sections, boards of education (school hygiene, physical education, and school lunch sections), and nursery schools through the heads (general affairs sections) of cities, towns, and villages, requesting each to evaluate whether they would cooperate. To those sections that were willing to cooperate, copies of a letter of request to parents, copies of the questionnaire, and envelopes were sent in necessary numbers. The answers were then gathered by each section and mailed to us. Cooperation in the survey was requested from the local governments of the 43 cities, towns, and villages in Osaka Prefecture, and one local government in each of the 47 prefectures in Japan between April and June, 2005. As a result, during the survey period between June and November, 2005, a total of 20,000 responses were collected in 30 cites, towns, and villages (recovery rate, roughly selected 75%). Of those responses which were finally compiled, those obtained from 20 randomly selected cities, towns, and villages (17,816 responses) are analyzed in this report. The responses concerning the vaccination rates and incidence rates (cumulative, within 1 year) were classified according to the vaccines used, the diseases, and age (Figs. 1–3).

From the results shown in Fig. 1, the prevalence of diseases that can be prevented by vaccination appears to be inversely correlated with the corresponding vaccination rates, since outbreaks of measles, rubella, and pertussis are controlled by vaccination. However, the vaccination rates against mumps and varicella have not reached a level effective for the control of outbreaks. Regarding influenza, although a considerable percentage of children were vaccinated, outbreaks recurred annually, and the number of patients

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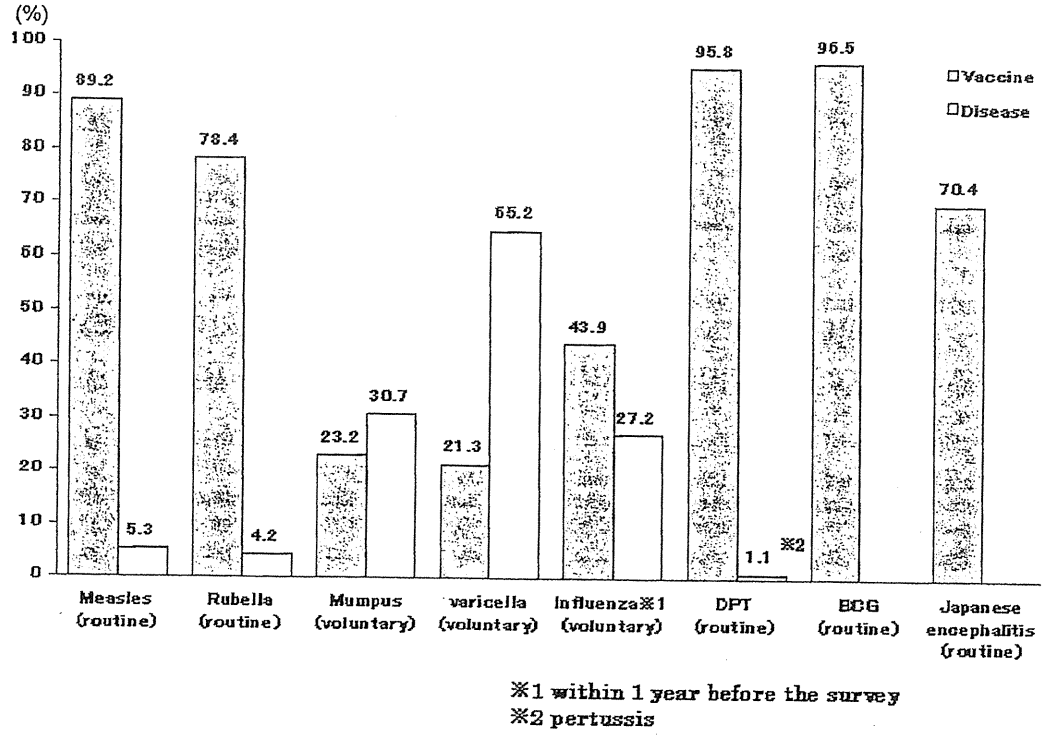
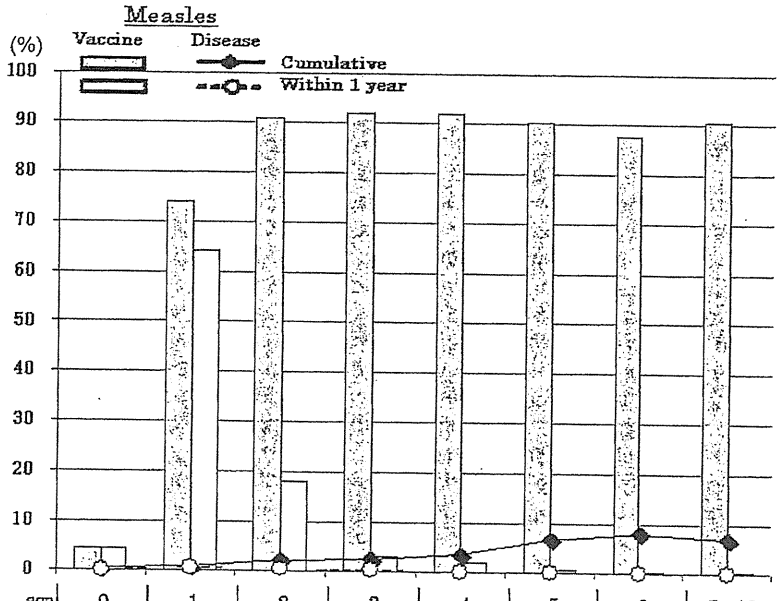


Fig. 1. Cumulative percentages of vaccination and natural infection (total number of 17,816 children aged 0–12 years in 20 cities, towns, and villages), in 2005 in Japan.

has not been reduced. The vaccination rate was 95% or higher only for BCG and DPT. The vaccination rates for other diseases, against which vaccination is performed routinely (Japanese encephalitis, measles, and rubella), were from 70 to 90%, although children will continue to contract these diseases given vaccination rates at this level. The vaccination rates for diseases against which vaccination is performed voluntarily (influenza, mumps, and varicella), are low, and outbreaks of these diseases are expected to persist at nursery schools (daycare centers), kindergartens, and elementary schools, probably explaining the high prevalence of these 3 diseases. Routine vaccination should be urgently instituted.

Vaccination rates against measles, along with its prevalence, prior to the introduction of the mixed measles–rubella (MR) vaccine, are shown in Fig. 2. The vaccination rate (cumulative) against measles did not exceed 90% until after the age of 2 years, and did not exceed 95% at any age level. As a result, children who had contracted measles were observed at all age levels. A rather higher percentage (64.2%) of children aged 1 year were vaccinated against



age	0	1	2	3	4	5	6	7~12
Vaccination rate	4.4(4.4)	73.9(64.2)	91.1(18.0)	92.1(3.1)	92.2(1.8)	90.4(0.6)	87.8(0.3)	90.8(0.3)
Incidence rate	0.5(0.0)	1.0(0.6)	2.3(0.4)	2.5(0.2)	3.5(0.0)	6.7(0.0)	8.0(0.1)	6.8(0.0)
Response number	181	798	1,091	2,359	2,330	2,675	1,593	6,599

cummulative (within 1 year)

Fig. 2. Vaccination rates against measles and its prevalence according to age (17,626 children aged 0–12 years in 20 cities, towns, and villages), in 2005 in Japan.