Table 2. HPV Type Prevalence in Japanese Women with Abnormal Cervical Cytology

HPV Types	Total (N = 326)		ASC-US (N = 59)		ASC-H (N = 22)		LSIL (N = 134)		HSIL (N = 86)		ICC (N = 25)	
	n*	%**	n*	%**	n*	%**	n*	%**	n*	%**	n*	%**
High-Risk												
16	69	21.2	9	15.3	5	22.7	14	10.4	31	36.0	10	40.0
18	23	7.1	1	1.7	1	4.5	9	6.7	8	9.3	4	16.0
26	1	0.3	0	0	0	0	0	0	1	1.2	0	0
31	24	7.4	3	5.1	3	13.6	5	3.7	10	11.6	3	12.0
33	6	1.8	0	0	0	0	2	1.5	3	3.5	1	4.0
35	6	1.8	0	0	0	0	3	2.2	3	3.5	0	0
39	16	4.9	3	5.1	0	0	11	8.2	1	1.2	1	4.0
45	1	0.3	0	0	0	0	0	0	0	0	1	4.0
51	24	7.4	2	3.4	3	13.6	11	8.2	8	9.3	0	0
52	66	20.2	12	20.3	9	40.9	22	16.4	21	24.4	2	8.0
53	23	7.1	6	10.2	1	4.5	12	9.0	2	2.3	2	8.0
56	27	8.3	3	5.1	1	4.5	20	14.9	3	3.5	0	0
58	48	14.7	8	13.6	4	18.2	23	17.2	12	14.0	1	4.0
59	10	3.1	3	5.1	1	4.5	4	3.0	2	2.3	0	0
66	15	4.6	1	1.7	0	0	13	9.7	1	1.2	0	0
68	5	1.5	1	1.7	0	0	2	1.5	2	2.3	0	0
73	1	0.3	1	1.7	0	0	0	0	0	0	0	0
82	6	1.8	1	1.7	0	0	1	0.7	4	4.7	0	0
Any***	281	86.2	44	74.6	20	90.9	111	82.8	83	96.5	23	92.0
Low-Risk												
6	8	2.5	1	1.7	0	0	4	3.0	3	3.5	0	0
11	1	0.3	1	1.7	0	0	0	0	0	0	0	0
34	1	0.3	0	0	0	0	1	0.7	0	0	0	0
40	0	0	0	0	0	0	0	0	0	0	0	0
42	9	2.8	3	5.1	0	0	5	3.7	1	1.2	0	0
43	0	0	0	0	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0	0	0	0	0
54	6	1.8	1	1.7	0	0	5	3.7	0	0	0	0
55	0	0	0	0	0	0	0	0	0	0	0	0
57	0	0	0	0	0	0	0	0	0	0	0	0
69	1	0.3	0	0	0	0	1	0.7	0	0	0	0
70	6	1.8	2	3.4	0	0	3	2.2	1	1.2	0	0
83	2	0.6	0	0	0	0	2	1.5	0	0	0	0
84	2	0.6	0	0	0	0	1	0.7	1	1.2	0	0
X	13	4.0	1	1.0	0	0	10	7.5	2	2.3	0	0
Any***	46	14.1	7	11.9	0	0	31	23.1	8	9.3	0	0
Overall	307	94.2	49	83.1	20	90.9	130	97.0	85	98.8	23	92.0

HPV genotypes are grouped based on the risk classification of Munoz *et al.* [3]. Type-specific prevalence includes HPVs in single or multiple infections.
*, Numbers of patients detected with each HPV.
**, Percentages relative to the total number of patients (N) in each cytological group.
***, Numbers of patients with any high- or low-risk HPVs and percentages relative to the total number of patients (N) in each cytological group.

adenocarcinoma, HPV16 was singly detected in 2 cases, and HPV18 was singly detected in 2 cases, whereas no HPV DNA was detected in one case.

DISCUSSION

This study provides the first data on the prevalence of HPV DNA and the distribution of HPV genotypes among

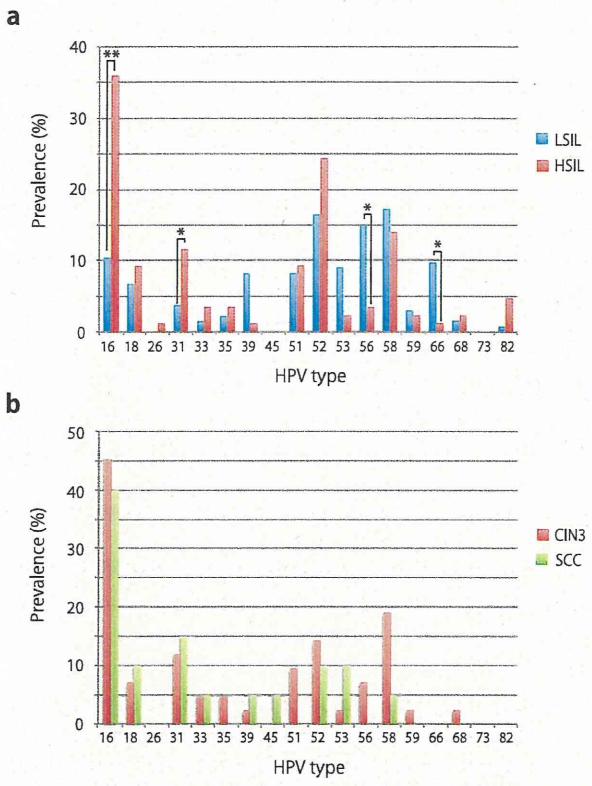

Fig. (2). Comparison of high-risk HPV type distribution in Japanese women with LSIL and HSIL (a), with CIN3 and SCC (b). * and ** indicate $P<0.05$ and $P<0.001$, respectively,

Japanese women with abnormal cervical smears using a highly sensitive genotyping assay that was recently validated by the HPV LabNet. The detection rates of HPV DNA were 97.0% (95% CI = 93.7% to 100%) in LSIL and 98.8% (95% CI = 92.5% to 99.2%) in HSIL, which are higher than those reported in a previous meta-analysis of studies in Japan: 79.4% in LSIL and 89.0% in HSIL [21]. With regard to multiple infections, the overall detection rate was 29.6% (95% CI = 24.6% to 35.1%) in women with cervical abnormalities, which is also higher than a previous report: 11.3 to 18.5% in Japanese CIN patients [15]. The increased detection rate of multiple infections likely reflects a higher sensitivity of the PGMY-CHUV assay to detect multiple infections without inter-type PCR competition compared to other genotyping methods used in previous studies in Japan, such as the L1C1/L1C2 primer system [13], and/or higher performance of AmpliTaq Gold® polymerase to amplify multiple HPV DNAs with the PGMY primers compared to other PCR enzymes [22].

The positivity of HPV DNA in ASC-US (83.1%, 95% CI = 71.0% to 91.2%) in this study seem to be higher than those reported in studies for cervical low-grade lesions in the US (61%) [23] and in Sweden (63%) [24]. This difference is likely due to the characteristics of our study population, which was not selected from primary screening programs but enrolled from outpatients mostly referred by primary care physicians due to abnormal cytology results. Furthermore, strict assignment of ASC-US cases in this study population, which is presumed to be relatively more HPV-positive than normal population, might have led to the high positivity of HPV DNA in ASC-US.

The previous meta-analysis of the HPV type prevalence in Japan showed that three most frequent genotypes in HSIL were HPV16 (34.3%), HPV52 (15.0%), and HPV58 (6.7%) [21]. In this study, the order of top three frequent HPV types in HSIL was the same with that reported in the meta-analysis, but more HPV52 (24.4%, 95% CI = 15.8% to 34.9%) and HPV58 (14.0%, 95% CI = 7.4% to 23.1%) were detected in this study. Given that the PGMY-CHUV assay exhibits a higher sensitivity and specificity for detection of HPV52 and 58 in multiple infection specimens [13], the previous surveys may have underestimated the prevalence of these genotypes. This may be also true of the HPV type distribution in LSIL cases. The meta-analysis revealed that five most frequent HPV types in LSIL were HPV16 (13.8%), HPV52 (11.1%), HPV51 (9.8%), HPV56 (9.0%), and HPV58 (5.8%) [21], but reported lower distribution rates for HPV52, 56, and 58, compared to those detected in this study: HPV52 (16.4%, 95% CI = 10.6% to 23.8%), HPV56 (14.9%,

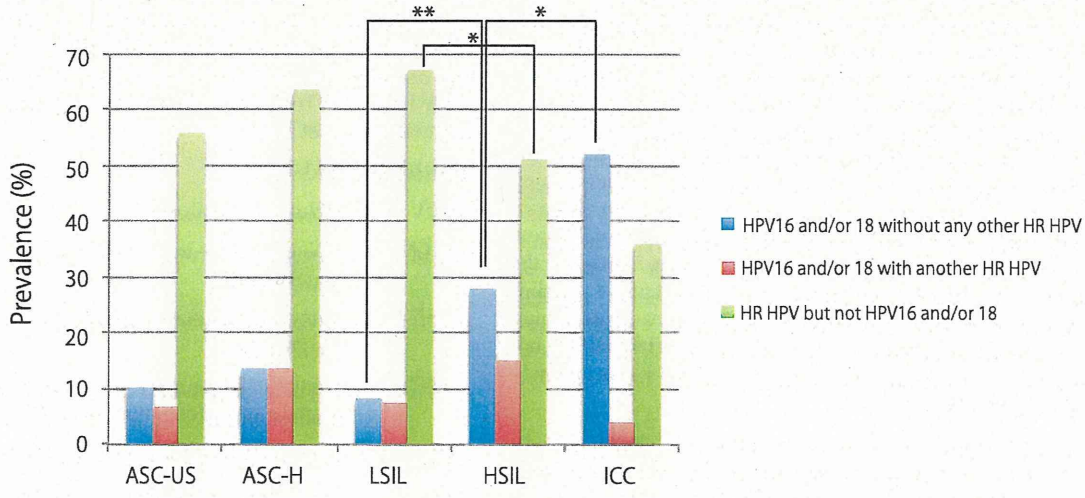

Fig. (3). Prevalence of HPV16 and/or 18 and other high-risk HPVs (alone or in mixed infections with HPV16 and/or 18). The percentages of cases are presented by each cervical abnormality grade. * and ** indicate $P<0.05$ and $P<0.001$, respectively,

Table 3. Multiple Infections (MI) of HPV in Japanese Women with Abnormal Cervical Cytology

	Total		ASC-US		ASC-H		LSIL		HSIL		ICC	
HPV (+)*	307		49		20		130		85		23	
MI*	91		12		8		42		27		2	
%**	29.6		24.5		40.0		32.3		31.8		8.7	
	n*	%**	n*	%**	n*	%**	n*	%**	n*	%**	n*	%**
1 type	216	70.4	37	75.5	12	60.0	88	67.7	58	68.2	21	91.3
2 types	62	20.2	8	16.3	7	35.0	28	21.5	17	20.0	2	8.7
3 types	22	7.2	2	4.1	1	5.0	11	8.5	8	9.4	0	0
4 types	6	2.0	2	4.1	0	0	3	2.3	1	1.2	0	0
5 types	1	0.3	0	0	0	0	0	0	1	1.2	0	0
MI of High-Risk HPVs												
	n*	%***	n*	%***	n*	%***	n*	%***	n*	%***	n*	%***
	77	84.6	8	66.7	8	100	35	83.3	24	88.9	2	100

*, Numbers of patients.
**, Percentages relative to the total number of HPV-positive patients in each cytological group.
***, Percentages relative to the total number of multiple-types infected patients in each cytological group.

95% CI = 9.4% to 22.1%), and HPV58 (17.2%, 95% CI = 11.2% to 24.6%).

In a meta-analysis using worldwide data on HPV type prevalence, a higher prevalence of HPV52 and 58 in HSIL and ICC was reported for East Asian countries, such as China, Taiwan, Japan, and Korea, compared to Europe, North America, and Africa [4,25], suggesting region-specific variation of HPV type prevalence in East Asia. Furthermore, a recent study by Takehara *et al.* [26] lends further support to the high prevalence of HPV52 and 58 in cervical intraepithelial lesions in Japanese women. Surprisingly, a recent clinical trial for the bivalent vaccine examining its cross-protective efficacy revealed negative vaccine efficacy for precancerous events associated with HPV52; vaccinated women developed slightly more HPV52-associated CIN2/3 lesions (excluding HPV16/18 co-infection) compared to the control women who received placebo [27]. Regarding an HPV genotyping method, it is worth noting that the InnoLiPA kit used in that trial was shown to be suboptimal for the detection of HPV52 in the global proficiency study of HPV genotyping [12], and thus may lead to underestimation of HPV52 in mixed infection samples, which indicates the necessity to confirm HPV52 prevalence by other typing methods or type-specific real-time PCR. Although the data are preliminary and will require further extensive evaluation, some potential for type-replacement, through which the protection of HPV16/18 infection by vaccines makes progression of HPV52-associated lesions more evident over time, warrants consideration, because high-risk HPVs not targeted by the vaccines tend to induce CIN3+ more slowly than HPV16/18 [28].

The HPV type distribution in CIN2/3 specimens of Japanese women was previously described in a study by Onuki *et al.* [15] as follows: three most frequent types were HPV16 (24.1%), HPV52 (17.5%), and HPV58 (10.7%). The higher detection rate of HPV16 (45.2%, 95% CI = 29.8% to 61.3%) in CIN3 specimens in this study, as well as that reported in previous epidemiological studies [14,15,28], strongly indicates a higher risk of HPV16 infection for progression to high-grade intraepithelial neoplasia and/or invasive cervical cancer.

Although the number of cervical cancer samples in this study was relatively small (n = 25), the detection rates of HPV vaccine-targeted types were 40% for HPV16 and 16% for HPV18, totaling 56% for both types, which lies in the range of 50 to 70% reported for cervical cancer in previous studies in Japan [7-9]. Further continuous investigation of the HPV type distribution in cervical cancer and its precursor lesions, such as CIN2/3 and adenocarcinoma in situ, in a central laboratory using a standardized genotyping assay will be required to monitor the efficacy of the current HPV vaccines, as well as to formulate a strategy for the development of the next generation HPV vaccines to meet the demand for protection from high-risk HPV infection other than HPV16/18 in each region, such as HPV52 and 58 in Japan and East Asia.

ABBREVIATIONS

HPV = Human papillomavirus

NILM = Negative for intraepithelial lesion or malignancy

ASC-US = Atypical squamous cells of undetermined significance

ASC-H = Atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion

LSIL = Low-grade squamous intraepithelial lesion

HSIL = High-grade squamous intraepithelial lesion

ICC = invasive cervical cancer

WHO = World Health Organization

CIN = Cervical intraepithelial neoplasia

SCC = Squamous cell carcinoma

CHUV = Centre Hospitalier Universitaire Vaudois

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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REFERENCES

[1] zur Hausen H, Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2002; (2): 342-50.

[2] de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. Virology 2004; 324: 17-27.

[3] Munoz N, Bosch FX, de Sanjose S, *et al.* Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003; 348: 518-27.

[4] Clifford GM, Smith JS, Aguado T, Franceschi S. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. Br J Cancer 2003; 89: 101-5.

[5] The FUTURE II Study Group Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions. N Engl J Med 2007; 356: 1915-27.

[6] Paavonen J, Naud P, Salmeron J, *et al.* Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women. Lancet 2009; 374: 301-14.

[7] Asato T, Maehama T, Nagai Y, *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.

[8] Nakagawa S, Yoshikawa H, Onda T, *et al.* Type of human papillomavirus is related to clinical features of cervical carcinoma. Cancer 1996; (78): 1935-41.

[9] 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.

[10] Estrade C, Menoud PA, Nardelli-Haefliger D, Sahli R. Validation of a low-cost human papillomavirus genotyping assay based on PGMY PCR and reverse blotting hybridization with reusable membranes. J Clin Microbiol 2011; 49: 3474-81.

[11] World Health Organization human papillomavirus laboratory manuaL, 1st ed. Available at: http://wwwwhoint/immunization/documents/WHO_IVB 2009;_1012/en/ (WNO/IVB/10.12) WHO, Geneva (Switzerland).

[12] Eklund C, Zhou T, Dillner J. Global proficiency study of human papillomavirus genotyping. J Clin Microbiol 2010; 48: 4147-55.

[13] Mori S, Nakao S, Kukimoto I, *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.

[14] Matsumoto K, Oki A, Furuta R, *et al.* Predicting the progression of cervical precursor lesions by human papillomavirus genotyping: a prospective cohort study. Int J Cancer 2011; 128: 2898-910.

[15] Onuki M, Matsumoto K, Satoh T, *et al.* Human papillomavirus infections among Japanese women: age-related prevalence and type-specific risk for cervical cancer. Cancer Sci 2009; 100: 1312-6.

[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] Yoshikawa H, Nagata C, Noda K, *et al.* Human papillomavirus infection and other risk factors for cervical intraepithelial neoplasia in Japan. Br J Cancer 1999; 80: 621-4.

[18] 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.

[19] Yoshida T, Fukuda T, Sano T, *et al.* Usefulness of liquid-based cytology specimens for the immunocytochemical study of p16 expression and human papillomavirus testing: a comparative study using simultaneously sampled histology materials. Cancer 2004; 102: 100-8.

[20] Nagai Y, Maehama T, Asato T, Kanazawa K. Persistence of human papillomavirus infection after therapeutic conization for CIN 3: is it an alarm for disease recurrence? Gynecol Oncol 2000; 79: 294-9.

[21] 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: 2713-15.

[22] Castle PE, Schiffman M, Gravitt PE, *et al.* Comparisons of HPV DNA detection by MY09/11 PCR methods. J Med Virol 2002; 68: 417-23.

[23] Castle PE, Solomon D, Schiffman M, Wheeler CM. Human papillomavirus type 16 infections and 2-year absolute risk of cervical precancer in women with equivocal or mild cytologic abnormalities. J Natl Cancer Inst 2005; 97: 1066-71.

[24] Brismar-Wendel S, Froberg M, Hjerpe A, Andersson S, Johansson B. Age-specific prevalence of HPV genotypes in cervical cytology samples with equivocal or low-grade lesions. Br J Cancer 2009; 101: 511-7.

[25] Smith JS, Lindsay L, Hoots B, *et al.* Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. Int J Cancer 2007; 121: 621-32.

[26] Takehara K, Toda T, Nishimura T, *et al.* Human papillomavirus types 52 and 58 are prevalent in uterine cervical squamous lesions from Japanese women. Patholog Res Int 2011; 2011: 246936.

[27] Wheeler CM, Castellsague X, Garland SM, *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.

[28] Kjaer SK, Frederiksen K, Munk C, Iftner T. Long-term absolute risk of cervical intraepithelial neoplasia grade 3 or worse following human papillomavirus infection: role of persistence. J Natl Cancer Inst 2010; 102: 1478-88.

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Monoclonal antibodies recognizing cross-neutralization epitopes in human papillomavirus 16 minor capsid protein L2

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ABSTRACT

Antisera induced by immunization of rabbits with the synthetic peptide P56/75, which has the amino acid (aa) sequence from aa56 to aa75 of HPV16 L2, neutralize pseudovirions and raft-virions of multiple high-risk HPV types, indicating that cross-neutralization epitopes are present in the aa56–75 region. We generated two mouse monoclonal antibodies (MAb): MAb13B and MAb24B recognizing the regions of aa64–73 and aa58–64, respectively. The neutralization assay using pseudovirions of HPV16, 18, 31, 33, 35, 51, 52 and 58 showed that MAb13B neutralized HPV16, 18, and 51, and MAb24B neutralized all the types tested. The mixture of MAb13B and MAb24B neutralized HPV16, 18, and 51 pseudovirions more efficiently than each of the MAbs alone. The data indicate that there are at least two cross-neutralization epitopes in the aa56–75 region and that an antigen capable of presenting the two cross-neutralization epitopes would be a good vaccine candidate for a broad-spectrum of HPVs.

Introduction

Human papillomavirus (HPV) is a small nonenveloped virus with the genome of an 8-kb double-stranded circular DNA (Howley and Lowy, 2001). The icosahedral capsid is composed of 360 molecules of major capsid protein L1 and 12 or more molecules of minor capsid protein L2 (Roden et al., 1996; Volpers et al., 1994; Buck et al., 2008). Based on the homology of nucleotide sequence of the L1 gene, HPVs are classified into more than 100 genotypes. Fifteen types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73) detectable in cervical cancer specimens are called high-risk types (Munoz et al., 2004). HPV type 16 (HPV16) is found in more than 50% of cervical cancer worldwide (Munoz et al., 2004).

Since cell cultures supporting efficient HPV replication are not available, some surrogate cell-HPV particle systems are used to analyze anti-HPV antibodies. L1 either alone or together with L2 expressed in cultured cells self-assembles into a virus-like particle (VLP) or an L1/L2-VLP, respectively, in the nucleus (Kirnbauer et al., 1992; Zhou et al., 1991). When L1/L2-VLP is formed in a cell harboring episomal copies of a reporter plasmid, the plasmid is packaged in the particle to form an infectious pseudovirion (PsV) (Buck et al., 2004; Stauffer et al., 1998; Unckell et al., 1997). Although it is not clear whether the L1/L2-VLP and the PsV are assembled in the same way as the authentic HPV virion, they are used as an antigen or surrogate virus for the detection of anti-HPV antibodies or neutralizing activity, respectively (Bontkes et al., 1999; Buck et al., 2005).

VLPs are highly immunogenic in animals and humans and are capable of inducing type-specific neutralizing antibodies (Giroglou et al., 2001; Harper et al., 2006; Villa et al., 2005). The vaccines using VLPs as antigens have been developed and licensed in many countries: the bivalent vaccine containing HPV16 and 18 VLPs (Cervarix™) and the tetravalent vaccine containing HPV6, 11, 16, and 18 VLPs (Gardasil™). Both vaccines efficiently induce type-specific neutralizing antibodies and protect the recipients from infection with HPVs of the vaccine-types (Harper et al., 2006; Villa et al., 2005). Thus, one of the remaining issues to be addressed would be the development of a prophylactic vaccine for a broad-spectrum of HPVs.

It has been shown that L2 of human or animal papillomaviruses elicits antibodies cross-neutralizing PsVs in vitro (Alphs et al., 2008; Embers et al., 2004; Gambhira et al., 2007a, 2007b; Kawana et al., 1999; Pastrana et al., 2005; Roden et al., 2000). We previously showed that antisera obtained by immunizing rabbits with a synthetic peptide having the amino acid (aa) sequence of

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HPV16 L2 from aa56 to aa75 (P56/75) neutralize the PsV of HPV16 and cross-neutralize those of HPV18, 31, and 58 (Kondo et al., 2007). Furthermore, it has been shown that anti-P56/75 neutralizes HPV16, 18, and 31 authentic virions derived from raft-cultures (Conway et al., 2009, 2011). These studies indicate that the L2 segment aa56 to aa75 should contain cross-neutralization epitopes.

Immunization of target animals with L2 peptides from some animal papillomaviruses (bovine and cottontail rabbit) protects the animals from experimental challenge with the corresponding papillomaviruses (Campo et al., 1997; Christensen et al., 1991; Embers et al., 2002; Gambhira et al., 2007a; Lin et al., 1992). Thus, the immunizations appear to work protectively like vaccination, despite apparent very low or often undetectable neutralizing titers of the serum anti-L2 antibodies of the protected animals probably due to the low sensitivity of the in vitro neutralization assays (Jagu et al., 2011). These findings provide a basis for the development of new HPV vaccines capable of inducing anti-L2 cross-neutralizing antibodies.

In this study we newly generated two hybridoma lines producing monoclonal antibodies (MAbs) with spleen cells of mice immunized with P56/75 and characterized the MAbs to know the location and the number of the cross-neutralization epitopes in the HPV16 L2 segment aa56 to aa75. The data indicate that there are at least two cross-neutralization epitopes in the region.

Results

Subtype and aa sequences of variable regions of the MAb

We immunized Balb/c mice with the synthetic peptide P56/75 which has the aa sequence from aa56 to aa75 of HPV16 L2, and obtained 24 hybridoma clones secreting MAbs capable of binding to P56/75. Two MAbs bound to HPV16 L1/L2-VLP as tested by ELISA but the others did not. The two MAbs, designated as MAb13B and MAb24B, were further characterized. Subtype of MAb13B was IgG1 and that of MAb24B was IgG2b.

Fig. 1 shows the aa sequences of their variable regions, which are deduced from the nucleotide sequences of the cDNA segments encoding the heavy- and light-chains.

Epitopes recognized by MAb13B and MAb24B

Fig. 2 shows levels of binding of the MAbs to the synthetic peptides having parts of the aa sequence of HPV16 L2 region from aa53 to aa75. The peptides conjugated with BSA were fixed to wells of an ELISA plate and then the purified MAb was added to the wells. The antibody bound to the peptide was detected by anti-mouse IgG goat serum.

MAb13B bound to P56/75, P53/73, PA58/75, PA61/75, and PA64/75, but not to P53/75A, P53/67A, and P53/65A. The data indicate that the epitope for MAb13B was present within the region from aa64 to aa73 of HPV16 L2.

MAb24B bound to P56/75, P53/73, P53/70A, PA53/67A, P53/64A, and PA58/75, but not to PA61/75 and PA64/75. The data indicate that the epitope for MAb24B was present within the region from aa58 to aa64 of HPV16 L2.

Binding of MAb13B and MAb24B to L1/L2 VLP

Fig. 3 shows levels of binding of the MAbs to the L1/L2-VLPs of HPV16, 18, 31, 33, 35, 51, 52, and 58. The serially diluted MAb (2000 to 2 ng) was allowed to react with the L1/L2-VLP (200 ng) fixed on wells of an ELISA plate. The antibody bound to the L1/L2-VLP was detected with anti-mouse IgG goat serum.

MAb13B bound to the L1/L2-VLPs of HPV16, 18, and 51 efficiently, and to those of HPV31, 33, and 58 less efficiently, but not to those of HPV35 and 52. MAb24B bound to the L1/L2-VLPs of all the types tested. MAb13B and MAb24B did not bound to VLP lacking L2 (data not shown), strongly suggesting that the MAbs bind to the L2 regions that are displayed on the surface of L1/L2-VLPs.

Neutralization of pseudovirions with MAb13B and MAb24B

Fig. 4 shows levels of neutralizing activity of the MAbs against the PsVs of HPV16, 18, 31, 33, 35, 51, 52, and 58. An anti-HPV16L1 monoclonal antibody, MAb25I, which neutralizes HPV16 PsV efficiently was used as a positive control. The two-fold serially diluted MAb solution, from 240 μg/ml to 0.47 μg/ml, was mixed with an equal volume of the suspension of infectious PsV that express secreted alkaline phosphatase (SEAP) and then the mixture was inoculated to 293FT cells. Three days later the SEAP activity of the culture medium was measured. The residual infectivity of the PsV mixed with the MAb was shown as a percentage of the SEAP activity to that obtained with the PsV without MAb. Table 1 shows the 50% inhibitory concentration (IC50) of the MAb calculated from the data.

MAb13B neutralized PsVs of HPV16, 18, and 51 with the IC50 of 1.9, 3.8, and 40.5 μg/ml, respectively, but did not neutralize PsVs of HPV31, 33, 35, 52, and 58. MAb24B neutralized PsVs of all

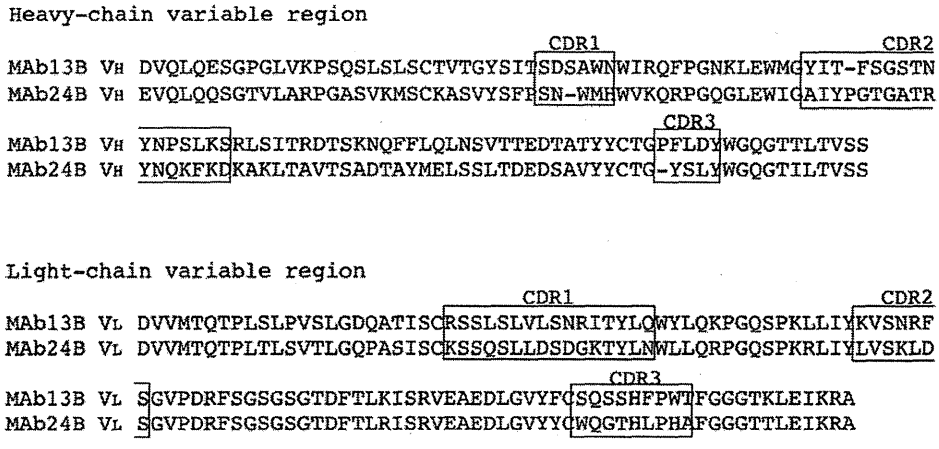

Fig. 1. Amino acid sequence of the variable region of MAb13B and MAb24B. cDNA encoding variable region of heavy chain (VH) or light chain (VL) of MAb13B and MAb24B was cloned and sequenced. Amino acid sequence was deduced from the nucleotide sequence. Complementarity-determining regions (CDRs) were boxed.

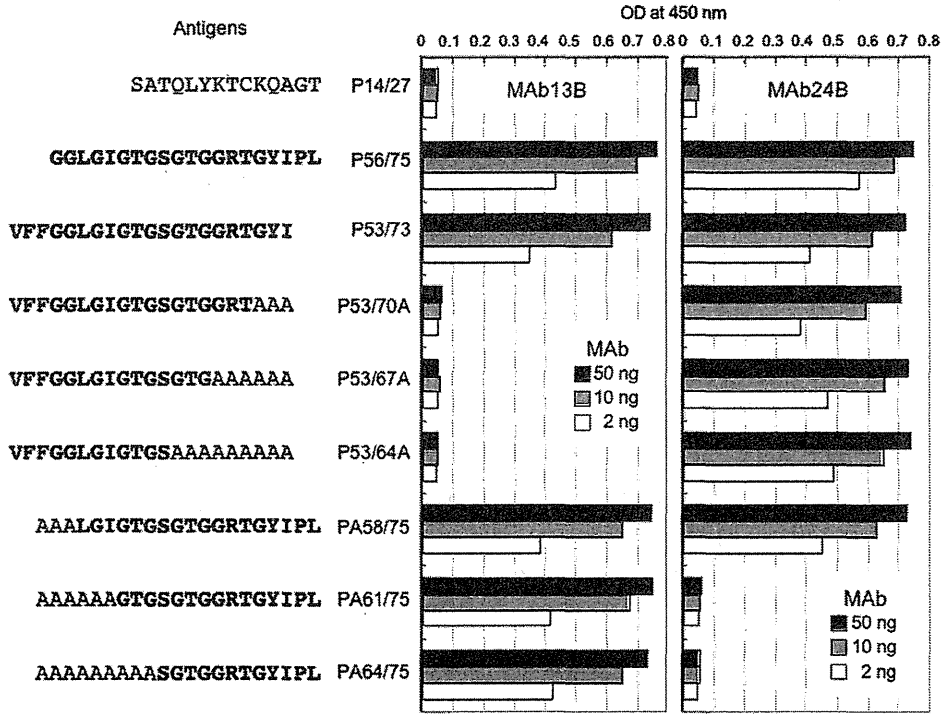

Fig. 2. Mapping of epitopes for MAb13B and MAb24B. The BSA-conjugated synthetic peptide (500 ng/well) was fixed on wells of an ELISA plate. The purified MAb13B (2, 10, or 50 ng/well) or MAb24B (2, 10, or 50 ng/well) was added to the wells and the antibody bound to the peptide was detected with anti-mouse IgG goat serum. The level of bound MAb is shown as absorbance at 450 nm.

the types tested. The mixture of equal amount of MAb13B and MAb24B (the total amount of the MAbs was indicated in Fig. 4 and Table 1) neutralized PsVs of HPV16, 18, and 51 (types neutralized with MAb13B) more efficiently than each MAb alone. The IC50 of the mixture for HPV16, 18, and 51 were two to five-fold lower than those of each MAb alone, indicating that the neutralization was enhanced synergistically.

The rabbit antibodies sharing binding sites with MAb13B and MAb24B

The rabbit anti-P56/75#1 serum (Kondo et al., 2007), which neutralizes HPV16, 18, and 31 authentic virions derived from raft-cultures (Conway et al., 2009, 2011), contained antibodies binding to the HPV16 L1/L2-VLP competitively with MAb13B and MAb24B (Fig. 5). The serially diluted anti-P56/75#1 was reacted with the HPV16 L1/L2-VLP fixed on wells of an ELISA plate. Then, MAb13B or MAb24B was allowed to bind to the antigen. Anti-P56/75#1 but not the preimmune serum inhibited the binding of MAb13B and MAb24B to the HPV16 L1/L2-VLP in a dose-dependent manner, suggesting that the epitopes recognized by the immunized rabbits (Kondo et al., 2007) were similar to those recognized by the immunized mice.

Discussion

This study indicates that at least two neutralization epitopes exist in the region from aa56 to aa75 of HPV16 L2. One epitope (for MAb13B) exists within the region from aa64 to aa73 (SGTGGRTGYI) and the other epitope (for MAb24B) exists within the region from aa58 to aa64 (LGIGTGS) (Figs. 2 and 6).

MAb13B bound to the L1/L2-VLPs of HPV16, 18 and 51 efficiently and neutralized pseudovirions of these types. HPV18 L2 has the region with the same aa sequence as that of the epitope for MAb13B. HPV51 L2 has the region with the aa sequence of SG**S**GGRTGYI, indicating that substitution of T (at aa66 of HPV16 L2) with **S** does not affect binding of the region with MAb13B.

MAb13B bound to the L1/L2-VLPs of HPV31, 33, and 58 less efficiently and did not neutralize pseudoviruions of these types. L2s of these types have the regions with aa sequences of the SG(T or **S**)GGRTGY**V**, indicating that substitution of I (at aa73 of HPV16 L2) with **V** reduces the affinity of the region for binding with MAb13B.

MAb13B did not bind to L1/L2-VLPs of HPV35 and 52. L2s of these types contain the regions with aa sequences of SG(T or **S**)GGR(**S** or **A**)GY**V**, indicating that MAb13B cannot bind to the region having further substitution of T (at aa70 of HPV16 L2) with **S** or with **A**. Thus, T and I (at aa70 and at aa73 of HPV16 L2, respectively) are critical for MAb13B to bind to L2 and neutralize PsV.

MAb24B bound to the L1/L2-VLPs of all HPV types tested and neutralized pseudovirions of these types. L2s of HPV18, 33, 51, 58 have the region with the aa sequence same as the epitope for MAb24B. HPV31 and 33 L2s have the region with aa sequences of LGIG**S**GS and HPV52 L2 has the region with aa sequence of LGIGTG**A**, indicating that substitution of T (at aa62 of HPV16 L2) with **S** and substitution of S (at aa64 of HPV16 L2) with **A** does not affect the binding of the regions with MAb24B.

The mixture of the half amounts of MAb13B and MAb24B (total amount of MAb was constant) neutralized HPV16, 18, and 51 more efficiently than each of the MAb alone, indicating that neutralization of these types was synergistically enhanced by the two MAbs (Table 1). It is possible that MAb13B and MAb24B bind to one molecule of L2 simultaneously, because the core regions of epitopes are not overlapping (Figs. 2 and 6). Probably the L2 making a complex with two antibodies greatly reduces its functions necessary for infection.

The rabbit anti-P56/75#1 serum (Kondo et al., 2007), which neutralizes HPV16, 18, and 31 authentic virions derived

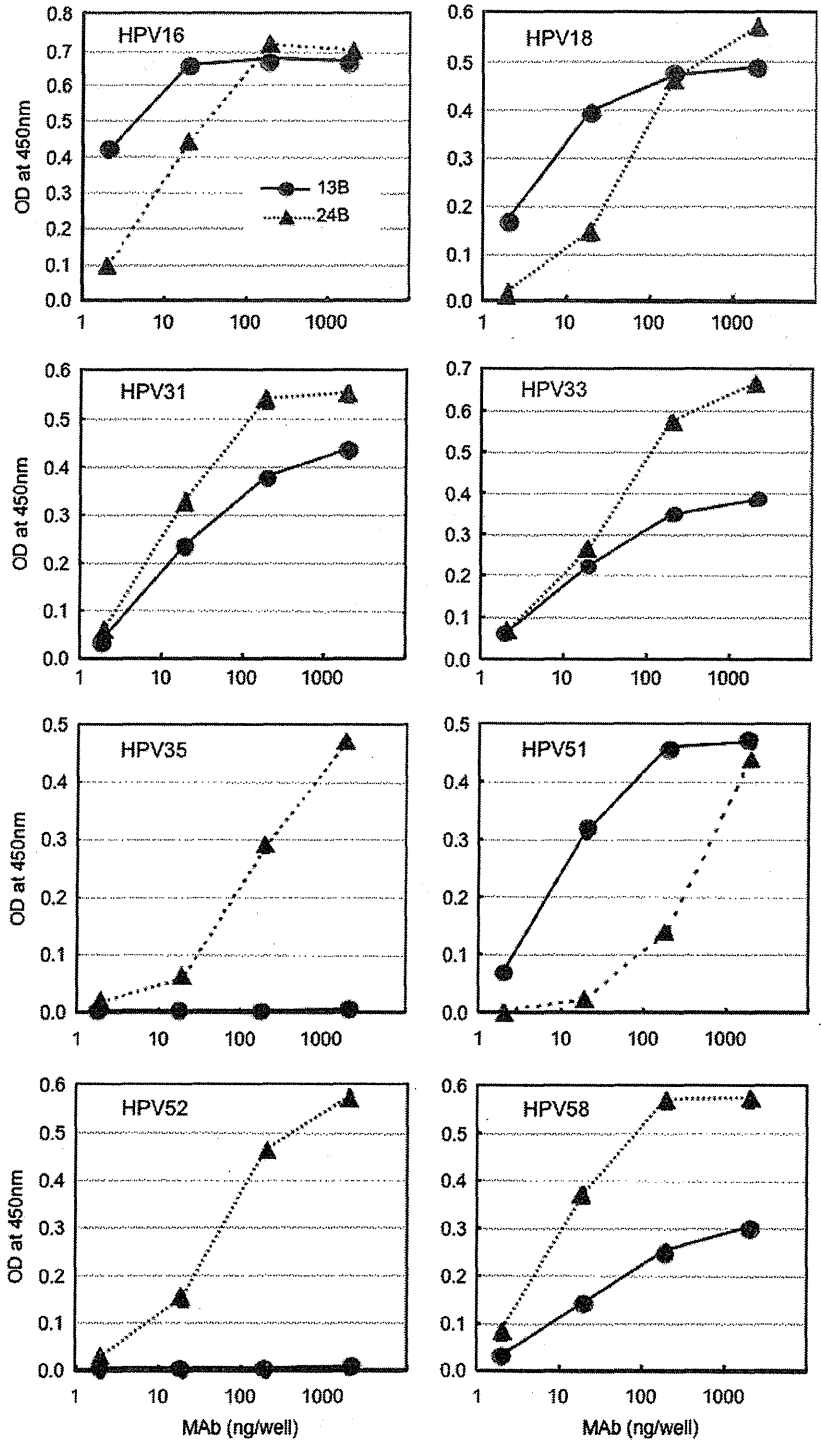

Fig.3. Binding of MAb13B and MAb24B to L1/L2-VLP. The purified L1/L2-VLP composed of L1 and L2 (200 ng/well) was immobilized on wells of an ELISA plate. The purified MAb13B (2, 20, 200, or 2000 ng/well) or MAb24B (2, 20, 200, or 2000 ng/well) was added to the well and the antibody bound to the L1/L2-VLP was detected with anti-mouse IgG goat serum. The level of bound MAb is shown as absorbance at 450 nm after subtraction of the background.

from raft-cultures (Conway et al., 2009, 2011), contained antibodies of which binding sites were overlapped with those of MAb13B and MAb24B (Fig. 5), indicating that the epitopes recognized by the rabbits are very similar to those recognized by the mice. It is possible that P56/75 may induce antibodies similar to MAb13B and MAb24B in humans.

Some cross-neutralizing anti-L2 MAbs were reported previously. MAb RG-1 recognizing the region from aa17 to aa36 of HPV16 L2 neutralized HPV16 and 18 PsVs (Gambhira et al., 2007b). MAbs recognizing the region from aa20 to aa38 of HPV16 L2 neutralized PsVs of HPV16, 18, 31, 45, 58, 57, and 27 (Rubio et al., 2011). These results, together with ours, strongly

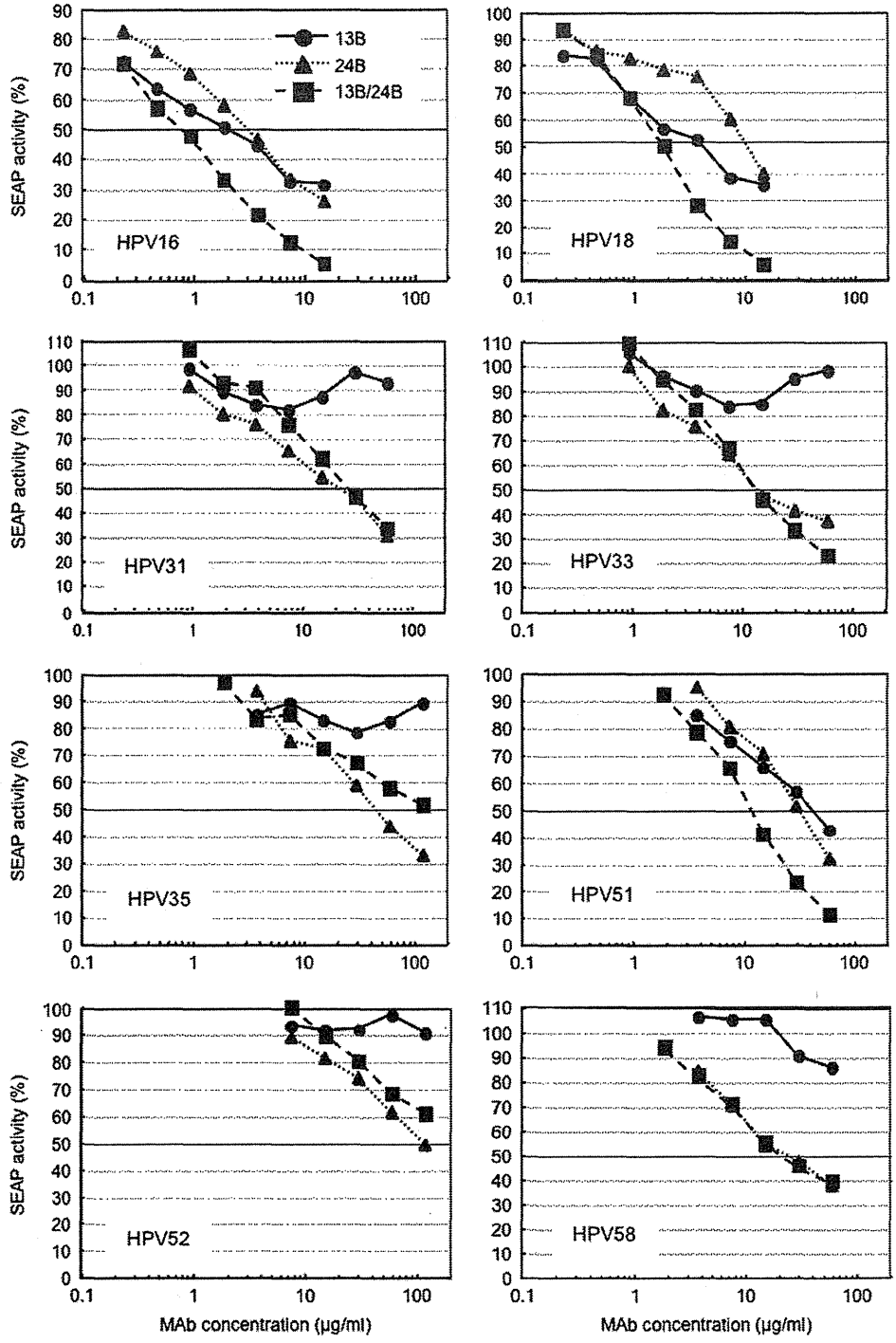

Fig. 4. Neutralization of pseudovirion (PsV) with MAb13B and MAb24B. The MAb solution was serially diluted two-fold and mixed with an equal volume of the PsV preparation, and then inoculated to 293FT cells. The cells infected with the PsV express secreted alkaline phosphatase (SEAP). Three days later the SEAP activity of the culture medium of the inoculated cells was measured and subtracted the background SEAP activity. The assay was done with triplicate. The percentage of the SEAP activity to that obtained without MAb was calculated and plotted. The final MAb concentration in the MAb/PsV mixture is presented.

suggest that the functions carried by the N-terminal region of HPV16 L2, from aa17 to aa75, are essential for infectivity of HPV and the functions may be interfered by the binding with antibodies. Because the aa sequences of the N-terminal regions of high-risk HPV L2s are similar, there are some epitopes consisting of the common aa sequences to multiple types. Again, the antigen capable of inducing antibodies recognizing such cross-neutralization epitopes may be a vaccine for multiple HPV types.

Materials and methods

Synthetic peptides

Nine peptides that mimic some HPV16L2 segments across aa53 to aa75 were synthesized by the Fmoc method by SCRUM Inc. (Tokyo, Japan) or BEX Co., LTD. (Tokyo, Japan). P56/75 or P53/73 represents a segment of aa56 to aa75 or aa53 to aa73,

Table 1
50% Inhibitory concentration (μg/ml) of MAb against PsV infection.

MAb	Genotype of pseudovirion							
	16	18	31	33	35	51	52	58
13B	1.9	3.8	120<	120<	120<	40.5	120<	120<
24B	3.0	11.7	21.4	16.0	47.3	30.8	118.1	25.0
13B/24B[a]	0.8[b]	1.8	24.4	14.4	120<	11.5	120<	26.1
25I[c]	0.006	10<	ND[d]	ND	ND	ND	ND	ND

[a] Mixture of equal amounts of MAb13B and MAb24B.
[b] Concentration of total MAb (combined MAb13B and MAb24B).
[c] MAb25I is an anti-HPV16L1 monoclonal antibody.
[d] Not done.

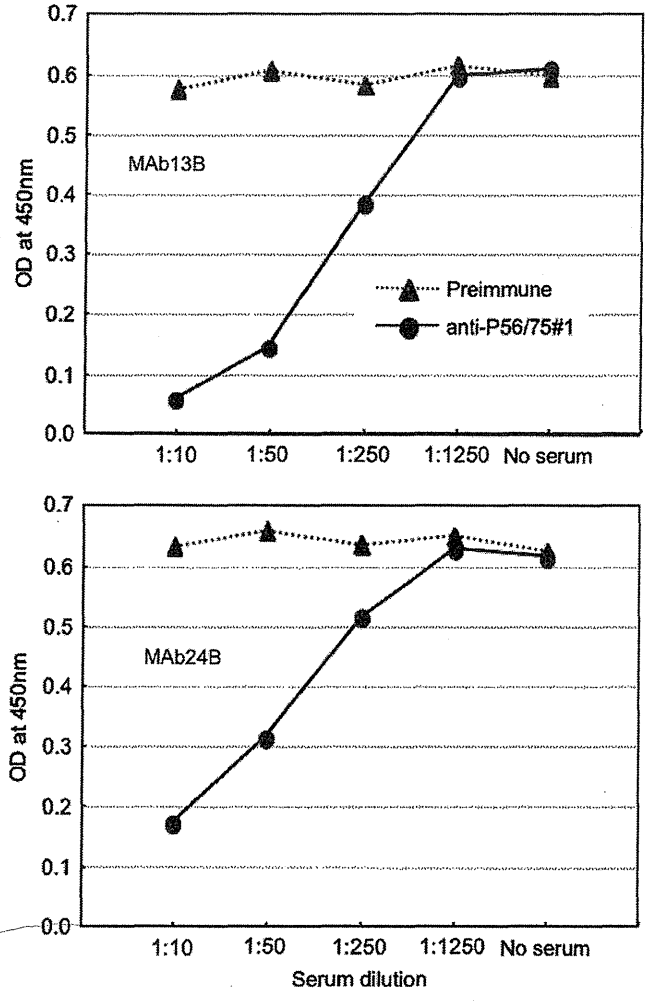

Fig. 5. Inhibition of the binding of MAb13B or MAb24B to HPV16 L1/L2-VLP by the rabbit anti-P56/75 serum. The serially diluted anti-P56/75#1 serum (Kondo et al., 2007) or preimmune rabbit serum was reacted with the HPV16 L1/L2-VLP (200 ng/well) fixed on wells of an ELISA plate. Then MAb13B (20 ng/well) or MAb24B (200 ng/well) was allowed to bind to the antigen and the MAb bound to the L1/L2-VLP was detected with anti-mouse IgG goat serum. The level of bound MAb is shown as absorbance at 450 nm after subtraction of the background.

respectively. P53/70A has a sequence of aa53 to aa70 plus three alanine residues. P53/67A has a sequence of aa53 to aa67 plus six alanine residues. P53/64A has a sequence of aa53 to aa64 plus nine alanine residues. PA58/75 has three alanine residues plus a sequence of aa58 to aa75. PA61/75 has six alanine residues plus a sequence of aa61 to aa75. PA64/75 has nine alanine residues plus a sequence of aa64 to aa75. P14/27, used as a negative control,

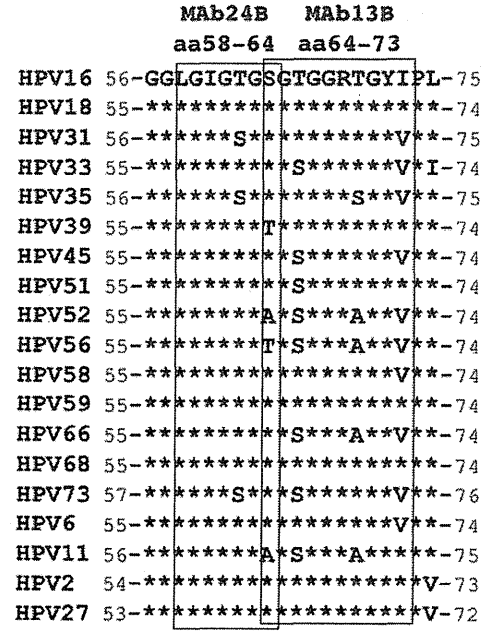

Fig. 6. Amino acid sequences of the region from aa56 to aa75 of HPV16 L2 and the corresponding regions of the other types. Amino acids identical to those of HPV16 L2 are indicated by asterisk (*). The regions containing the epitopes for MAb13B and MAb24B are boxed.

has the sequence of aa14 to aa27. Cysteine was added to the N-terminus of the peptides to conjugate with keyhole limpet hemocyanin (KLH) or with bovine serum albumin (BSA).

MAb

Six-weeks-old BALB/c mice (3 animals) were immunized with a mixture of P56/75 conjugated with KLH (50 μg/animal) and Freund's complete adjuvant subcutaneously 4 times at an interval of 2 weeks. At 1 week after the fourth immunization, mice were injected with 25 μg of P56/75 intraperitoneally. Splenocytes harvested from mice at 1 week after the booster injection were fused with mouse myeloma cells, P3U1, by the standard polyethylene glycol method. The hybridomas secreting MAb capable of binding to P56/75 conjugated with BSA were selected and then those secreting MAb capable of binding to HPV16 L1/L2-VLP were further selected. MAb25I is an anti-HPV16L1 monoclonal antibody isolated from a BALB/c mouse immunized with HPV16L1-VLP and neutralized HPV16 PsV (our unpublished data).

MAb was purified from the ascites, which was obtained from mice injected with the hybridoma intraperitoneally, by using Ab-Rapid SPiN column (ProteNova, Takamatsu, Japan). The MAb was eluted from the beads and dialyzed against phosphate-buffered saline (PBS). The resultant MAb solution was used in the analyses. The protein concentration of the MAb solution was measured by comparison with standard solutions of BSA on an SDS-PAGE gel. The subtype of the MAb was determined by the Mouse Monoclonal Antibody Isotyping Test Kit (AbD Serotec, Oxford, UK), according to the manufacturer's instruction.

Rabbit antiserum

Rabbit anti-P56/75 #1 serum was described in the previous study (Kondo et al., 2007). The serum was obtained from the rabbit subcutaneously injected with a mixture of P56/75 conjugated with KLH and Freund's complete adjuvant 4 times at 2-week interval.

VLP and L1/L2-VLP

Expression plasmids for HPV16 L1 (p16L1h), HPV16 L2 (p16L2h), HPV18 L1 (peL1fB), and HPV18 L2 (peL2bhb) were gifts from J.T. Schiller (NCI, USA). Expression plasmids for HPV31 L1 (p31L1h), HPV31 L2 (p31L2h), HPV51 L1 (p51L1h), HPV51 L2 (p51L2h), HPV52 L1 (p52L1h), HPV52 L2 (p52L2h), HPV58 L1 (p58L1h), and HPV58 L2 (p58L2h) were constructed previously (Kondo et al., 2007, 2008, 2009). Expression plasmids for HPV33L1 (p33L1h), HPV33 L2 (p33L2h), HPV35 L1 (p35L1h), and HPV35 L2 (p35L2h) were similarly constructed in this study.

293FT cells (Life Technologies Corp.), a human kidney cell line expressing SV40 T-antigen, were transfected with an L1-plasmid (30 μg) for production of VLP or with a mixture of an L1-plasmid (24.5 μg) and an L2-plasmid (5.5 μg) for production of the L1/L2-VLP by using FuGENE HD (Rosch Diagnostics GmbH, Mannheim, Germany). Fifty-eight hours later the VLP or the L1/L2-VLP was extracted from the cells and purified as described previously (Kondo et al., 2007).

Cloning and nucleotide sequencing of immunoglobulin variable regions

Total RNA was extracted from the hybridoma by using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). The cDNA segments encoding variable regions of light chain and heavy chain of immunoglobulin were amplified by SMARTer RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA) with primers for the constant regions of IgG1, IgG2b, and kappa chain, whose nucleotide sequences had been described previously (Wang et al., 2000). PCR products were cloned into pGEM-T Easy vector (Promega Corporation, Madison, WI) and sequenced with an ABI 3730 Genetic Analyzer (Life Technologies Corp., Carlsbad, CA). Consensus sequences of 8 clones obtained by independent PCR amplification were adopted. The amino acid sequence was deduced from the nucleotide sequence.

ELISA

The purified VLP (200 ng) or the synthetic peptide conjugated with BSA (500 ng) in 50 μl of PBS was added to wells of an ELISA plate (Thermo Labsystems, Franklin, MA) and incubated for overnight at 4 °C. After washings with PBS containing 0.1% Tween-20 (Washing buffer), the wells were blocked with 5% skim milk in PBS containing 0.1% Tween-20 (Blocking buffer) for 2 h at room temperature (RT). The MAb diluted in Blocking buffer was added to the wells and incubated for 2 h at RT. The horseradish peroxidase-conjugated anti-mouse IgG goat serum (SC-2031, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted at 1:2000 with Blocking buffer was added to the well and incubated for 1 h at RT. After washings with Washing buffer a mixture of 0.01% H_2O_2 and o-phenylenediamine (2 mg/ml) in 0.1 M citrate buffer (pH4.7) was added to the wells and the absorbency at 450 nm was measured.

Neutralization of PsV with MAb

The codon modified L1 and L2 genes of HPV16, 18, 31, 33, 35, 51, 52, and 58 were inserted into the multicloning sites A and B of pIRES vector (Clontech Laboratories, Inc.), respectively, to obtain plasmids (approximately 9 kb in size), pIRESL1/L2-16, -18, -31, -33, -35, -51, -52, and -58. An expression plasmid for SEAP (pYSEAP) was gifts from J.T. Schiller (NCI, USA).

293FT cells were transfected with a mixture of pIRESL1/L2 plasmid (16.5 μg) and pYSEAP (13.5 μg). Two days later the PsV was extracted and purified from the cells using "Alternative Protocol" described at URL: http://home.ccr.cancer.gov/lco/rip cord.htm.

The neutralization test was carried out as described previously (Kondo et al., 2008). Briefly the mixture of serially diluted MAb (two-fold dilution starting from 120 μg/ml) and PsV suspension was incubated for 1 h at 4 °C and then inoculated to 293FT cells. Three days later the SEAP activity of the culture medium was measured by the colorimetric SEAP assay. The background OD value was subtracted from each measurement. The relative to the maximum OD, the measurement of the culture medium of the cells inoculated with PsV without MAb, was calculated (%) and plotted (Fig. 4). The MAb concentration that reduced OD to 50% of the maximum was calculated from the total MAb concentration in the MAb/PsV mixture and linear percent inhibition regression line developed and presented as IC50 in Table 1.

Disclosure statement

S. Mori and T. Kanda were government employees when they produced MAb13B and MAb24B. The Japan Health Sciences Foundation, which deals with inventions by government employees, applied for a patent on the method measuring anti-L2 neutralizing antibodies that recognize epitopes overlapping with those recognized by MAb13B and MAb24B. S. Mori and T. Kanda are the inventors of the patent.

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References

Alphs, H.H., Gambhira, R., Karanam, B., Roberts, J.N., Jagu, S., Schiller, J.T., Zeng, W., Jackson, D.C., Roden, R.B., 2008. Protection against heterologous human papillomavirus challenge by a synthetic lipopeptide vaccine containing a broadly cross-neutralizing epitope of L2. Proc. Natl. Acad. Sci. USA 105, 5850–5855.

Bontkes, H.J., De Gruijl, T.D., Walboomers, J.M., Schiller, J.T., Dillner, J., Helmerhorst, T.J., Verheijen, R.H., Scheper, R.J., Meijer, C.J., 1999. Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia. II. Systemic but not local IgA responses correlate with clearance of HPV-16. J. Gen. Virol. 80, 409–417.

Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T., 2004. Efficient intracellular assembly of papillomaviral vectors. J. Virol. 78, 751–757.

Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T., 2005. Generation of HPV pseudovirions using transfection and their use in neutralization assays. Methods Mol. Med. 119, 445–462.

Buck, C.B., Cheng, N., Thompson, C.D., Lowy, D.R., Steven, A.C., Schiller, J.T., Trus, B.L., 2008. Arrangement of L2 within the Papillomavirus Capsid. J. Virol. 82, 5190–5197.

Campo, M.S., O'Neil, B.W., Grindlay, G.J., Curtis, F., Knowles, G., Chandrachud, L., 1997. A peptide encoding a B-cell epitope from the N-terminus of the capsid protein L2 of bovine papillomavirus-4 prevents disease. Virology 234, 261–266.

Christensen, N.D., Kreider, J.W., Kan, N.C., DiAngelo, S.L., 1991. The open reading frame L2 of cottontail rabbit papillomavirus contains antibody-inducing neutralizing epitopes. Virology 181, 572–579.

Conway, M.J., Alam, S., Christensen, N.D., Meyers, C., 2009. Overlapping and independent structural roles for human papillomavirus type 16 L2 conserved cysteines. Virology 393, 295–303.

Conway, M.J., Cruz, L., Alam, S., Christensen, N.D., Meyers, C., 2011. Cross-neutralization potential of native human papillomavirus N-terminal L2 epitopes. PLoS One 6, e16405.

Embers, M.E., Budgeon, L.R., Culp, T.D., Reed, C.A., Pickel, M.D., Christensen, N.D., 2004. Differential antibody responses to a distinct region of human papillomavirus minor capsid proteins. Vaccine 22, 670–680.

Embers, M.E., Budgeon, L.R., Pickel, M., Christensen, N.D., 2002. Protective immunity to rabbit oral and cutaneous papillomaviruses by immunization with short peptides of L2, the minor capsid protein. J. Virol. 76, 9798–9805.

Gambhira, R., Jagu, S., Karanam, B., Gravitt, P.E., Culp, T.D., Christensen, N.D., Roden, R.B., 2007a. Protection of rabbits against challenge with rabbit papillomaviruses by immunization with the N terminus of human papillomavirus type 16 minor capsid antigen L2. J. Virol. 81, 11585–11592.

Gambhira, R., Karanam, B., Jagu, S., Roberts, J.N., Buck, C.B., Bossis, I., Alphs, H., Culp, T., Christensen, N.D., Roden, R.B., 2007b. A protective and broadly cross-neutralizing epitope of human papillomavirus L2. J. Virol. 81, 13927–13931.

Giroglou, T., Sapp, M., Lane, C., Fligge, C., Christensen, N.D., Streeck, R.E., Rose, R.C., 2001. Immunological analyses of human papilloma virus capsids. Vaccine 19, 1783–1793.

Harper, D.M., Franco, D.M., Wheeler, C.M., Moscocki, A.B., Romanoswski, B., Roteli-Martins, C.M., Jenkins, D., Schuind, A., Costa Clemens, S.A., Dubin, G., 2006. HPV vaccine study group. 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 367, 1247–1255.

Howley, P.M., Lowy, D.R., 2001. Papillomaviruses and their replication. 4th ed. In: Lnipe, D.M., Howley, P.M. (Eds.), Fields Virology, vol. 2. Lippincott, Williams and Wilkins, Philadelphia, pp. 2197–2229.

Jagu, S., Malandro, N., Kwak, K., Yuan, H., Schlegel, R., Palmer, K.E., Huh, W.K., Campo, M.S., Roden, R.B., 2011. A multimeric L2 vaccine for prevention of animal papillomavirus infections. Virology 420, 43–50.

Kawana, K., Yoshikawa, H, Taketani, Y., Kanda, T., 1999. Common neutralizing epitope in minor capsid protein L2 of human papillomavirus Types 16 and 6. J. Virol. 73, 6188–6190.

Kirnbauer, R., Booy, F., Cheng, N., Lowy, D.R., Schiller, J.T., 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc. Natl. Acad. Sci. USA 89, 12180–12184.

Kondo, K., Ishii, Y., Mori, S., Shimabukuro, S., Yoshikawa, H., Kanda, T., 2009. Nuclear location of minor capsid protein L2 is required for expression of a reporter plasmid packaged in HPV51 pseudovirions. Virology 394, 259–265.

Kondo, K., Ishii, Y., Ochi, H., Matsumoto, T., Yoshikawa, H., Kanda, T., 2007. Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. Virology 358, 266–272.

Kondo, K., Ochi, H., Matsumoto, T., Yoshikawa, H., Kanda, T., 2008. Modification of human papillomavirus-like particle vaccine by insertion of the cross-reactive L2-epitopes. J. Med. Virol. 80, 841–846.

Lin, Y.L., Borenstein, L.A., Selvakumar, R., Ahmed, R., Wettstein, F.O., 1992. Effective vaccination against papilloma development by immunization with L1 or L2 structural protein of cottontail rabbit papillomavirus. Virology 187, 612–619.

Munoz, N., Bosch, F.X., Castellsague, X., Diaz, M., Sanjose, S., Hammouda, D., Shah, K.V., Meijer, C.J.L.M., 2004. Against which human papillomavirus types shall we vaccinate and screen? Int. J. Cancer 111, 278–285.

Pastrana, D.V., Gambhira, R., Buck, C.B., Pang, Y.Y.S., Thompson, C.D., Culp, T.D., Christensen, N.D., Lowy, D.R., Schiller, J.T., Roden, R.B.S., 2005. Cross-neutralization of cutaneous and mucosal papillomavirus types with anti-sera to the amino terminus of L2. Virology 337, 365–372.

Roden, R.B., Greenstone, H.L., Kirnbauer, R., Booy, F.P., Jessie, J., Lowy, D.R., Schiller, J.T., 1996. In vitro generation and type-specific neutralization of a human papillomavirus type 16 virion pseudotype. J. Virol. 70, 5875–5883.

Roden, R.B., Yutzy, W.H., Fallon, R., Inglis, S., Lowy, D.R., Schiller, J.T., 2000. Minor capsid protein of human genital papillomaviruses contains subdominant, cross-neutralizing epitopes. Virology 270, 254–257.

Rubio, I., Seitz, H., Canali, E., Sehr, P., Bolchi, A., Tommasino, M., Ottonello, S., Müller, M., 2011. The N-terminal region of the human papillomavirus L2 protein contains overlapping binding sites for neutralizing, cross-neutralizing and non-neutralizing antibodies. Virology 409, 348–359.

Stauffer, Y., Raj, K., Masternak, K., Beard, P., 1998. Infectious human papillomavirus type18 pseudovirions. J. Mol. Biol. 283, 529–536.

Unckell, F., Streeck, R.E., Sapp, M., 1997. Generation and neutralization of pseudovirions of human papillomavirus type 33. J. Virol. 71, 2934–2939.

Villa, L.L., Costa, R.L., Petta, C.A., Andrade, R.P., Ault, K.A., Giuliano, A.R., Wheeler, C.M., Koutsky, L.A., Malm, C., Lehtinen, M., Skjeldestad, F.F., Olsson, S.E., Steinwall, M., Brown, D.R., Kurman, R.J., Ronnett, B.M., Stoler, M.H., Ferenczy, A., Harper, D.M., Tamms, G.M., Yu, J., Lupinacci, L., Railkar, R., Taddeo, F.J., Jansen, K.U., Esser, M.T., Sings, H.L., Saah, A.J., Barr, E., 2005. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncol. 6, 256–257.

Volpers, C., Schirmacher, P., Streeck, R.E., Sapp, M., 1994. Assembly of the major and the minor capsid protein of human papillomavirus type 33 into virus-like particles and tubular structures in insect cells. Virology 200, 504–512.

Wang, Z., Raifu, M., Howard, M., Smith, L., Hansen, D., Goldsby, R., Ratner, D., 2000. Universal PCR amplification of mouse immunoglobulin gene variable regions: the design of degenerate primers and an assessment of the effect of DNA polymerase 3′ to 5′ exonuclease activity. J. Immunol. Methods 233, 167–177.

Zhou, J., Sun, X.Y., Stenzel, D.J., Frazer, I.H., 1991. Expression of vaccinia recombinant HPV16L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. Virology 185, 251–257.

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NOTE

Novel multiplexed genotyping of human papillomavirus using a VeraCode-allele-specific primer extension method

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ABSTRACT

A VeraCode-allele-specific primer extension (ASPE) method was applied to the detection and genotyping of human papillomavirus (HPV)-DNA. Oligonucleotide primers containing HPV-type-specific L1 sequences were annealed to HPV-DNA amplified by PGMY-PCR, followed by ASPE to label the DNA with biotinylated nucleotides. The labeled DNA was captured by VeraCode beads through hybridization, stained with a streptavidin-conjugated fluorophore, and detected by an Illumina BeadXpress® reader. By using this system, 16 clinically important HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) were correctly genotyped in a multiplex format. The VeraCode-ASPE genotyping of clinical DNA samples yielded identical results with those obtained by validated PGMY-reverse blot hybridization assay, providing a new platform for high-throughput genotyping required for HPV epidemiological surveys.

Key words allele-specific primer extension, genotyping, human papillomavirus, multiplex assay.

Human papillomaviruses (HPV) are recognized as the causative agents of cervical cancer, its precursor lesions, and other anogenital cancers (1). Among more than 100 HPV types so far identified, nearly 40 types infecting the anogenital mucosa are classified as either low- or high-risk types on the basis of their oncogenic potentials (2). A previous large-scale case–control study revealed 15 high-risk types, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82, which are closely linked to the development of cervical cancer, with HPV16 the predominant high-risk type worldwide (3). In contrast, low-risk HPV types, including HPV6 and 11, are associated almost exclusively with benign lesions. Due to the lack of a cell culture system to isolate HPV from clinical samples, detection of HPV-DNA is the only reliable means for diagnosis of HPV infection. HPV genotyping is of particular importance for understanding the natural history of HPV infection and management of cervical cancers. In addition, with the worldwide introduction of HPV vaccines that target the two prominent high-risk types, HPV16 and 18, there is a growing demand for reliable and practical HPV genotyping to monitor HPV prevalence and vaccine efficacy at both individual and population levels.

Various molecular techniques have been developed for detection of HPV-DNA, most of which rely on amplification of HPV-DNA by PCR. The PCR of HPV-DNA generally utilizes degenerate/consensus primer systems, such as MY09/11 (4), PGMY09/11 (5), GP5+/6+ (6), or SPF (7), all of which are designed to amplify the L1 region of the HPV genome. For HPV genotyping, PCR is followed

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List of Abbreviations: ASPE, allele-specific primer extension; HPV, human papillomavirus; MFI, median fluorescence intensity; RBH, reverse blot hybridization; RLB, reverse line blot; SNP, single nucleotide polymorphism; WHO, World Health Organization.

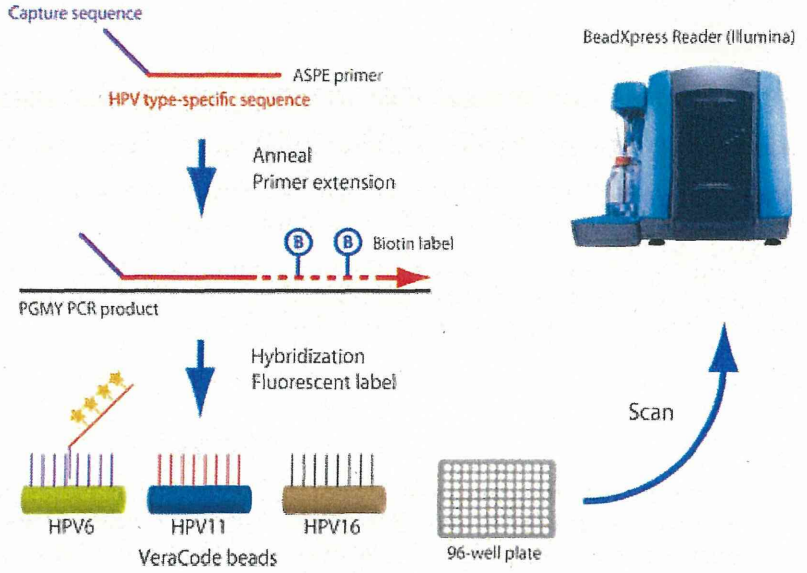

Fig. 1. Outline of VeraCode-ASPE HPV genotyping. HPV-type-specific ASPE primers are annealed to PGMY-PCR products, followed by primer extension by DNA polymerase to label DNA with biotin-14-dCTP. The biotin-labeled DNA is hybridized with VeraCode beads via a capture sequence at the 5′ end of the ASPE primer, and labeled with a streptavidin-fluorophore conjugate to allow detection of fluorescent signals by a BeadXpress® reader in a 96-well plate format. Each VeraCode bead contains digitally assigned barcodes so that the BeadXpress® reader can identify HPV-type-specific signals from each VeraCode-bead, enabling HPV genotyping with digital read-out. The BeadXpress® reader photograph is used with the permission of Illumina Inc. (San Diego, CA, USA).

by sequence analysis, restriction fragment length polymorphism analysis, or hybridization with type-specific oligonucleotide probes by a membrane-based RLB assay. Of the various HPV genotyping assays, the RLB assay has the advantage of being able to detect multiple HPV-type infections with greater sensitivity. Several RLB assays combined with different PCR schemes have been established and used for HPV research and diagnostic purposes (8–10). However, the RLB assays are relatively laborious, are limited to a maximum of about 40 samples per assay, and depend on visual read-out of the hybridization signal. To overcome these drawbacks, HPV genotyping using Luminex® suspension array technology has been developed (11–14). The Luminex®-based genotyping coupled with GP5+/6+ PCR allowed sensitive and specific genotyping of 27 mucosal HPV types in a 96-well plate format with a digital read-out (13). Moreover, a modified version of GP5+/6+ PCR was successfully introduced into the Luminex®-based assay, and showed improved sensitivity (15).

A VeraCode-ASPE method was first developed for the detection of SNP in the human genome (16) and has been applied to multiplex SNP genotyping on the Illumina BeadXpress® platform (17, 18). The ASPE primer is composed of two distinct regions: the 5′ region that contains the capture sequence, which is used in a subsequent hybridization reaction, and the 3′ region that contains the genomic target region with a SNP nucleotide at the extreme 3′ end. For SNP genotyping, the ASPE primer that matches the SNP nucleotide to the genome is extended by the primer extension reaction and is thus labeled with biotinylated nucleotides. After the primer extension, the products are mixed with VeraCode beads, so that the capture sequence on the primer hybridizes to its complementary sequence attached to the VeraCode beads. Labeling is then carried out with a streptavidin-fluorophore conjugate, followed by scanning and detection of the fluorescent signal using an Illumina BeadXpress® reader (Illumina Inc., San Diego, CA, USA).

In this work, the VeraCode-ASPE method on the Illumina BeadXpress® platform was evaluated for its suitability as a method to detect and genotype HPV-DNA (Fig. 1). The HPV-DNA amplified by PGMY-PCR was selected as a target for the VeraCode-ASPE genotyping, as PGMY-PCR has been validated as a sensitive and specific means for HPV-DNA amplification (19, 20). HPV-type-specific ASPE primers were designed to target the PCR amplicons of 16 HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) in the 3′ region (Table 1), and with type-specific capture sequences in the 5′ region. The T_m values of the HPV-type-specific sequences, the lengths of which ranged from 19 to 28 bases, were adjusted to be between 54°C and 66°C using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) thus allowing similar annealing profiles.

HPV-DNA, which was provided by the HPV laboratory network in the WHO as a quality-assured authentic panel for validation of HPV genotyping, was used to assess the sensitivity and specificity of the VeraCode-ASPE HPV genotyping. Fifty copies of HPV16- and 18-DNA and 500 copies of the other 14 HPV-type DNAs from the panel were subjected to PGMY-PCR with AmpliTaq Gold® polymerase (Applied Biosystems, Foster City, CA,

Table 1. HPV-type-specific sequences in ASPE primers for VeraCode-ASPE genotyping

Type	DNA sequence (5′–3′)	Tm (°C)
HPV6	CATCCGTAACTACATCTTCCA	55.3
HPV11	GCATCTGTGTCTAAATCTGCTAC	55.9
HPV16	AGTACCTACGACATGGGGAG	56.6
HPV18	CCAGGTACAGGAGACTGTGTA	55.3
HPV31	TGCAATTGCAAACAGTGATAC	57.3
HPV33	CTTTATGCACACAAGTAACTAGTGA	55.7
HPV35	TGTTCTGCTGTGTCTTCTAGTGA	57.8
HPV39	CCAACTTTACATTATCTACCTCTATAGA	55.3
HPV45	CACAAAATCCTGTGCCAAGT	58.6
HPV51	ACTGCTGCGGTTTCCCCAA	65.6
HPV52	GGAATACCTTCGTCATGGC	57.9
HPV56	TGATGCACGAAAAATTAATCAG	57.9
HPV58	TGACATTATGCACTGAAGTAACTAAG	57.0
HPV59	AAAGAATATGCCAGACATGTG	55.3
HPV66	AGTTAATGTGCTTTTAGCTGC	54.3
HPV68	GCTGTACCAAATATTTATGATCCTAA	57.1

USA) as described (21). One-third of the PCR products was treated with 2 U shrimp alkaline phosphatase and 5 U exonuclease I at 37°C for 45 min, followed by the ASPE reaction in a mixture containing 1× PCR buffer II (Roche, Indianapolis, IN, USA), 2.5 mM $MgCl_2$, 5 μM of each dATP, dGTP and dTTP, 7.5 μM biotin-14-dCTP, 0.05 μM of each ASPE primer, 0.5 U AmpliTaq Gold® polymerase, with denaturation at 95°C for 10 min followed by 50 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 45 sec. The reaction products were then incubated with the VeraCode bead mixture for 1 hr at 45°C in a VeraCode-bead plate, followed by staining with streptavidin-Alexa-647 in a buffer consisting of 3× standard saline citrate (SSC) and 0.1% Tween 20 for 15 min at room temperature. The VeraCode-bead plate was subjected to scanning by the BeadXpress® reader, and the read-out was expressed as the MFI obtained from each HPV type-assigned bead. As shown in Figure 2a, the 16 types of HPV-DNA were specifically detected with signals from their corresponding VeraCode beads. Signal values from non-target HPV-DNAs were as low as those from DNA-negative samples, and were classified as background noises. Furthermore, when the panel DNA containing a mixture of HPV-DNA was analyzed, corresponding signals from included HPV types were correctly detected (Fig. 2b), which indicates that VeraCode-ASPE typing is applicable to the simultaneous detection of multiple HPV-type DNAs.

To test the suitability of this assay for diagnostic purposes, DNA samples prepared from clinical specimens were analyzed by VeraCode-ASPE HPV genotyping. DNA was purified using the QIAamp® DNA blood kit (QIAGEN, Hilden, Germany) from cervical exfoliated cells that

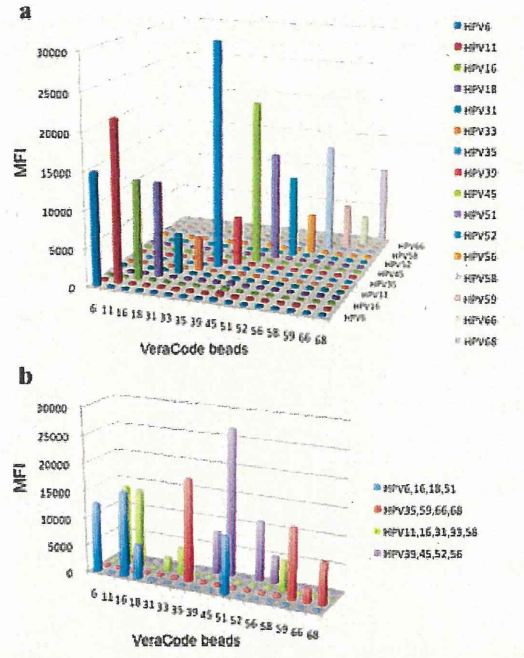

Fig. 2. VeraCode-ASPE genotyping of the WHO HPV-DNA proficiency panel. (a) PGMY-PCR products derived from the panel DNA containing each single-type HPV-DNA were subjected to the VeraCode-ASPE reaction and analyzed by the BeadXpress® reader. The signals from each VeraCode bead are shown as the MFI. The VeraCode bead number indicates the HPV type number that is assigned to each bead. (b) PGMY-PCR products derived from the panel DNA containing multiple-type HPV-DNA were subjected to the VeraCode-ASPE reaction and analyzed by the BeadXpress® reader.

had been collected from outpatients with their informed consent for HPV genotyping. The study design was approved by the institutional review board of the NTT Medical Center, Tokyo. DNA samples were previously genotyped by PGMY-reverse blot hybridization (PGMY-RBH) assay, which had been validated as to be sensitive and specific for genotyping of the 16 HPV types in the studies of the WHO HPV-DNA proficiency panel (20). The same PGMY-PCR products derived from these DNA samples were subjected to VeraCode-ASPE HPV genotyping as carried out for the WHO HPV-DNA panel. A positive result was defined as a signal value more than three-fold the average background value for each HPV-type-specific VeraCode bead. Of 50 clinical samples analyzed by the VeraCode-ASPE assay, 20 samples gave HPV-positive results, whereas the remaining 30 samples were judged to be negative. Table 2 shows raw MFI data and typing results of the VeraCode-ASPE assay with 20 positive samples and one negative sample. Overall, the typing results were identical to those obtained by the PGMY-RBH assay, which strongly suggests that the VeraCode-ASPE assay can

Table 2. VeraCode-ASPE genotyping of DNA from clinical specimens

DNA no.	HPV6 HPV45 Typing result	HPV11 HPV51	HPV16 HPV52	HPV18 HPV56	HPV31 HPV58	HPV33 HPV59	HPV35 HPV66	HPV39 HPV68
#1644	46	50	47	102	53	47	53	78
	58	**28537**	50	51	48	71	424	147
	HPV51							
#1646	233	185	209	298	194	207	204	224
	213	320	237	**14999**	243	215	672	262
	HPV56							
#1647	203	237	**14047**	281	219	215	191	240
	217	297	200	224	217	**19118**	647	307
	HPV16, 59							
#1650	221	243	214	296	246	255	221	231
	253	323	**12271**	260	251	229	669	333
	HPV52							
#1652	47	54	51	140	57	46	56	97
	59	140	**24978**	55	56	55	608	173
	HPV52							
#1654	201	212	186	316	206	202	216	258
	821	289	219	191	**14963**	197	743	312
	HPV45, 58							
#1657	389	383	318	434	353	363	334	**1570**
	363	445	**46218**	395	364	346	825	431
	HPV39, 52							
#1661	120	130	128	238	166	136	149	**4599**
	156	221	151	134	123	137	622	255
	HPV39							
#1662	242	221	**45495**	244	186	223	238	270
	229	279	**26066**	209	230	231	518	286
	HPV16, 52							
#1666	204	252	**2654**	301	236	191	225	265
	223	330	226	243	**16990**	225	556	**5996**
	HPV16, 58, 68							
#1668	81	83	**38858**	135	92	84	86	121
	95	148	**10338**	84	94	83	435	178
	HPV16, 52							
#1669	118	156	122	189	**7566**	129	148	151
	143	207	140	131	**30234**	151	449	224
	HPV31, 58							
#1672	209	209	214	281	220	203	170	241
	219	266	216	201	**22802**	218	578	290
	HPV58							
#1676	91	92	96	169	96	87	92	121
	100	**12816**	89	99	96	97	491	190
	HPV51							
#1679	279	274	240	**36487**	238	268	278	270
	257	325	293	232	276	265	519	354
	HPV18							
#1683	241	278	**39325**	312	276	232	246	266
	249	321	251	233	258	226	552	337
	HPV16							
#1684	91	87	104	130	88	87	94	103
	96	145	89	101	86	91	**26638**	160
	HPV66							
#1685	151	129	**6983**	237	150	122	137	174
	150	221	146	145	145	139	559	233
	HPV16							

Continued.

Table 2. Continued

DNA no.	HPV6 HPV45 Typing result	HPV11 HPV51	HPV16 HPV52	HPV18 HPV56	HPV31 HPV58	HPV33 HPV59	HPV35 HPV66	HPV39 HPV68
#1686	196	198	198	289	169	204	235	203
	208	276	202	187	186	**20644**	574	275
	HPV59							
#1691	230	205	230	346	227	234	216	222
	250	275	225	**13139**	226	241	552	317
	HPV56							
#1645	150	138	142	252	151	136	148	183
	147	219	143	148	134	131	659	229
	negative							
DNA(–)	188	205	202	289	195	198	213	211
	217	264	206	220	204	222	457	259
Cut-off	565	615	607	868	584	595	638	634
	650	791	618	659	611	667	1372	778

Signal values above cut-off values are indicated in bold letters.

substitute for the reverse blot hybridization on the same platform of PGMY-PCR.

The principle of the allele-specific primer extension was previously used in tag-array-based HPV genotyping (22, 23); however, the array format of this assay hampers its application to high-throughput HPV genotyping. In contrast, the 96-well plate format of the VeraCode-ASPE method enables HPV genotyping for large amounts of clinical samples. Furthermore, there are a total of 144 different sets of VeraCode beads, and thus it is possible to include more HPV types in the VeraCode-ASPE genotyping format. In conclusion, the VeraCode-ASPE genotyping is a powerful new tool for the high-throughput HPV genotyping that will be required for large-scale surveillance of HPV-type distribution at the population level in the near future.

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DISCLOSURE

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REFERENCES

1. Zur Hausen H. (2002) Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* **2**: 342–50.
2. De Villiers E.M., Fauquet C., Broker T.R., Bernard H.U., Zur Hausen H. (2004) Classification of papillomaviruses. *Virology* **324**: 17–27.
3. Munoz N., Bosch F.X., De Sanjose S., Herrero R., Castellsague X., Shah K.V., Snijders P.J., Meijer C.J. (2003) Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* **348**: 518–27.
4. Coutlee F., Hankins C., Lapointe N. (1997) Comparison between vaginal tampon and cervicovaginal lavage specimen collection for detection of human papillomavirus DNA by the polymerase chain reaction. The Canadian Women's HIV Study Group. *J Med Virol* **51**: 42–7.
5. Gravitt P.E., Peyton C.L., Alessi T.Q., Wheeler C.M., Coutlee F., Hildesheim A., Schiffman M.H., Scott D.R., Apple R.J. (2000) Improved amplification of genital human papillomaviruses. *J Clin Microbiol* **38**: 357–61.
6. De Roda Husman A.M., Walboomers J.M., Van Den Brule A.J., Meijer C.J., Snijders P.J. (1995) The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *J Gen Virol* **76**(Pt 4): 1057–62.
7. Kleter B., Van Doorn L.J., Ter Schegget J., Schrauwen L., Van Krimpen, K., Burger M., Ter Harmsel B., Quint W. (1998) Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. *Am J Pathol* **153**: 1731–39.
8. Van Den Brule A.J., Pol R., Fransen-Daalmeijer N., Schouls L.M., Meijer, C.J., Snijders P.J. (2002) GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. *J Clin Microbiol* **40**: 779–87.
9. Gravitt P.E., Peyton C.L., Apple R.J., Wheeler, C.M. (1998) Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. *J Clin Microbiol* **36**: 3020–27.

10. Kleter B., Van Doorn, L.J., Schrauwen L., Molijn A., Sastrowijoto S., Ter Schegget J., Lindeman J., Ter Harmsel B., Burger M., Quint W. (1999) Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. *J Clin Microbiol* **37**: 2508–17.
11. Schmitt M., Bravo I.G., Snijders P.J., Gissmann L., Pawlita M., Waterboer T. (2006) Bead-based multiplex genotyping of human papillomaviruses. *J Clin Microbiol* **44**: 504–12.
12. Oh Y., Bae S.M., Kim Y.W., Choi H.S., Nam G.H., Han S.J., Park C.H., Cho Y., Han B.D., Ahn W.S. (2007) Polymerase chain reaction-based fluorescent Luminex assay to detect the presence of human papillomavirus types. *Cancer Sci* **98**: 549–54.
13. Schmitt M., Dondog B., Waterboer T., Pawlita M. (2008) Homogeneous amplification of genital human alpha papillomaviruses by PCR using novel broad-spectrum GP5+ and GP6+ primers. *J Clin Microbiol* **46**: 1050–59.
14. Nazarenko I., Kobayashi L., Giles J., Fishman C., Chen G., Lorincz A. (2008) A novel method of HPV genotyping using Hybrid Capture sample preparation method combined with GP5+/6+ PCR and multiplex detection on Luminex XMAP. *J Virol Methods* **154**: 76–81.
15. Soderlund-Strand A., Carlson J., Dillner J. (2009) Modified general primer PCR system for sensitive detection of multiple types of oncogenic human papillomavirus. *J Clin Microbiol* **47**: 541–46.
16. Lin C.H., Yeakley J.M., Mcdaniel T.K., Shen R. (2009) Medium- to high-throughput SNP genotyping using VeraCode microbeads. *Methods Mol Biol* **496**: 129–42.
17. Tokarska M., Marshall T., Kowalczyk R., Wojcik J.M., Pertoldi C., Kristensen T.N., Loeschcke V., Gregersen V.R., Bendixen C. (2009) Effectiveness of microsatellite and SNP markers for parentage and identity analysis in species with low genetic diversity: the case of European bison. *Heredity* **103**: 326–32.
18. Mefford H.C., Cooper G.M., Zerr T., Smith J.D., Baker C., Shafer N., Thorland E.C., Skinner C., Schwartz C.E., Nickerson D.A., Eichler E.E. (2009) A method for rapid, targeted CNV genotyping identifies rare variants associated with neurocognitive disease. *Genome Res* **19**: 1579–85.
19. Mori S., Nakao S., Kukimoto I., Kusumoto-Matsuo R., Kondo K., Kanda T. (2011) Biased amplification of human papillomavirus DNA in specimens containing multiple human papillomavirus types by PCR with consensus primers. *Cancer Sci* **102**: 1223–7.
20. Estrade C., Menoud P.A., Nardelli-Haefliger D., Sahli R. (2011) Validation of a low-cost HPV genotyping assay baced on PGMY-PCR and reverse blotting hybridization with reusable membranes. *J Clin Microbiol* **49**: 3474–81.
21. Unger E.R., Dillner J., Zhou T. (2009) *Human Papillomavirus Laboratory Manual*, 1st edition. Geneva, Switzerland: WHO.
22. Kaller M., Hultin E., Zheng B., Gharizadeh B., Wallin K.L., Lundeberg J., Ahmadian A. (2005) Tag-array based HPV genotyping by competitive hybridization and extension. *J Virol Methods* **129**: 102–12.
23. Gheit T., Landi S., Gemignani F., Snijders P.J., Vaccarella S., Franceschi S., Canzian F., Tommasino M. (2006) Development of a sensitive and specific assay combining multiplex PCR and DNA microarray primer extension to detect high-risk mucosal human papillomavirus types. *J Clin Microbiol* **44**: 2025–31.

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Genotype-Dependent Efficacy of a Dual PI3K/mTOR Inhibitor, NVP-BEZ235, and an mTOR Inhibitor, RAD001, in Endometrial Carcinomas

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Abstract

The PI3K (phosphatidylinositol-3-kinase)/mTOR (mammalian target of rapamycin) pathway is frequently activated in endometrial cancer through various PI3K/AKT-activating genetic alterations. We examined the antitumor effect of NVP-BEZ235—a dual PI3K/mTOR inhibitor—and RAD001—an mTOR inhibitor—in 13 endometrial cancer cell lines, all of which possess one or more alterations in *PTEN*, *PIK3CA*, and *K-Ras*. We also combined these compounds with a MAPK pathway inhibitor (PD98059 or UO126) in cell lines with *K-Ras* alterations (mutations or amplification). *PTEN* mutant cell lines without *K-Ras* alterations (n = 9) were more sensitive to both RAD001 and NVP-BEZ235 than were cell lines with *K-Ras* alterations (n = 4). Dose-dependent growth suppression was more drastically induced by NVP-BEZ235 than by RAD001 in the sensitive cell lines. G1 arrest was induced by NVP-BEZ235 in a dose-dependent manner. We observed in vivo antitumor activity of both RAD001 and NVP-BEZ235 in nude mice. The presence of a MEK inhibitor, PD98059 or UO126, sensitized the *K-Ras* mutant cells to NVP-BEZ235. Robust growth suppression by NVP-BEZ235 suggests that a dual PI3K/mTOR inhibitor is a promising therapeutic for endometrial carcinomas. Our data suggest that mutational statuses of *PTEN* and *K-Ras* might be useful predictors of sensitivity to NVP-BEZ235 in certain endometrial carcinomas.

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Introduction

Constitutive activation of the PI3K (phosphatidylinositol 3-kinase) pathway results from various types of alterations, including changes to RTKs (receptor tyrosine kinases), *Ras*, *PIK3CA* (the p110alpha catalytic subunit of PI3K), and *PTEN* [1]. Endometrial cancer is the fourth most frequent cancer in women [2]. There are two pathogenetic types of endometrial carcinomas: estrogen-dependent type I (endometrioid adenocarcinomas) and estrogen-independent type II (high-grade carcinomas). Approximately 80% of endometrial carcinomas are classified as type I [3,4]. Mutations of *K-Ras* (10–20%), *PTEN* (34–56%), and *PIK3CA* (25–36%) are frequently observed in endometrial cancer [5–8]. In addition, we previously revealed that chromosomal imbalances in the Ras-PI3K pathway genes (*NF1*, *PTEN*, *K-Ras*, and *PIK3CA*) are also common in endometrial cancer [9], indicating that the Ras-PI3K pathway is activated in the majority of endometrial cancers.

Novel therapeutics targeting the PI3K/mTOR (mTORC1/2) pathway are being intensively developed. The first clinically approved inhibitors are rapamycin analogs (rapalogs), such as everolimus (RAD001) and temsirolimus, targeting the mTORC1 complex for use with advanced renal cell carcinomas [10–13]. However, clinical trials with single-agent rapalog therapies have shown limited response rates in other cancer types [14]. Several potent and selective PI3K inhibitors have recently entered early-phase clinical trials for treatment of various malignant tumors [15]. The limitation of the rapalogs might be explained by the activity of the mTORC1-independent substrates of Akt, including GSK3beta and FOXO1/3a. Rapalogs do not prevent mTORC2-dependent phosphorylation of Akt on Ser-473 or PDK1-dependent phosphorylation of Akt on Thr-308 [16,17]. In addition, rapalogs may cause feedback activation of the PI3K-Akt pathway mediated by insulin-like growth factor-1 receptor (IGF-1R) signaling [18]. Therefore, a dual PI3K/mTOR inhibition might be a more rational therapeutic option than mTOR inhibition alone in tumors with PI3K-activating mutations.

Developing predictive biomarkers of the PI3K/mTOR inhibitors is important; however, the existence of alterations in the PI3K

pathway (or elevated AKT phosphorylation) alone is not necessarily a good biomarker for these compounds. Indeed, tumors with alterations in Ras and RTK do not respond sufficiently to simple PI3K pathway inhibition [19–22]. Moreover, multiple genetic alterations in the RTK-Ras-PI3K pathway are reported in many cancers [1]. It remains to be determined which types of alterations are useful as predictive biomarkers.

In this study, we firstly evaluated the antitumor effect of a dual PI3K/mTOR inhibitor, NVP-BEZ235, and an mTOR inhibitor, RAD001 (everolimus), in a panel of endometrial cancer cell lines. Second, we analyzed the antitumor effect of NVP-BEZ235 and RAD001 in vivo. Third, we focused on the predictive biomarkers to the PI3K/mTOR inhibitors, using the mutational status of *K-Ras*, *PTEN*, and *PIK3CA*. Finally, we addressed the antitumor effect of the combined inhibition of the PI3K/mTOR and MAPK pathways in cells with *K-Ras* alterations.

Materials and Methods

Cell lines and reagents

Culture conditions of 13 endometrial cancer cell lines (endometrioid adenocarcinomas) were described previously [8]. NVP-BEZ235 and RAD001 (everolimus) were kindly provided by Novartis Pharma AG (Basel, Switzerland). MAPK pathway (MEK) inhibitors PD98059 and UO126 were purchased from Cell Signaling Technology (Beverly, MA).

PCR and sequencing

The mutational status of 13 cell lines was analyzed by PCR and direct sequencing. The PCR conditions and primers for *PTEN* (exons 1–9), *K-Ras* (exon 1 and 2), and *AKT1* (exon 4) were described previously [8,23,24]. The mutational status of *PIK3CA* was analyzed by RT-PCR with LA-Taq according to the manufacturer's protocol (Takara BIO, Madison, WI) to cover entire coding region. The PCR primers were the following: forward, 5′-CCCGAGCGTTTCTGCTTTGGGACAACC-3′; reverse, 5′-AGCGTTTCTGCTTTGGGACAACCATACATC-3′.

Immunoblotting

Cells were treated with each drug for the indicated time and concentrations and then lysed as described previously [7]. Antibodies to total Akt, phospho-Akt (Ser473), phospho-Akt (Thr308), phospho-GSK3beta (Ser9), total S6, phospho-S6 (Ser235/236, Ser 240/244), p-4EBP1 (Thr37/46), total FoxO1, phospho-FoxO1 (Thr24), phospho-FoxO3a (Thr32), phospho-ERK (ERK1/2-Thr202/Tyr204), total ERK (Cell Signaling Technology, Beverly, MA), and beta-actin (Sigma-Aldrich, St. Louis, MO) were used for immunoblotting, as recommended by the manufacturer, and were detected by an ECL western blot detection kit (Amersham Biosciences, Piscataway, NJ) or Immobilon western detection reagents (Millipore Biosciences, Temecula, CA).

Proliferation assays

Cell viability assays were performed with the Cell Counting Kit-8 (cell viability colorimetric assay), using the tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Dojindo, Tokyo, Japan), as an MTT (Methyl thiazolyl tetrazorium) assay. In 96-well plates, 1×10^5 cells were seeded with 10% fetal bovine serum (FBS) and treated with increasing doses of NVP-BEZ235 or RAD001 for 72 h, starting 24 h after seeding. The WST-8 colorimetric assay was quantified at 415 nm and normalized to the value of cells treated with DMSO alone. All experiments were repeated twice.

Cell cycle analysis

Cells (5×10^5) were seeded in 60-mm dishes (with 10% FBS) and treated with reagents (such as NVP-BEZ235, RAD001, PD98059, or UO126) for 48 h. Floating and adherent cells were collected by trypsinization and washed twice with PBS. Cells were resuspended in buffer containing ethanol and PBS at a ratio of 7:3 at −20°C overnight. After being washed twice with PBS, cells were incubated in ribonuclease solution (0.25 mg/mL) (Sigma) for 30 min at 37°C, followed by staining with propidium iodide (50 μg/mL) (Dojindo, Japan) on ice for 30 min in the dark. Cells were then incubated in 70% ethanol at −20°C overnight, treated with 20 μg/mL RNase A, stained with 0.5 μg/mL propidium iodide, and evaluated by flow cytometry (BD FACS Calibur HG, Franklin Lakes, NJ). Cell cycle distribution was analyzed with CELL Quest pro ver. 3.1. (Beckman Coulter Epics XL, Brea, CA). The experiments were repeated 3 times.

Ethics statement for animal experiments and clinical data

Ethics statement for animal experiments: This study was approved by Animal Care and Use Committee, The University of Tokyo. The approval number is Med-P09-051. Athymic BALB/c mice (CLEA JAPAN, Tokyo, Japan) were maintained in an SPF (Specific Pathogen Free) facility according to our institutional guidelines, and experiments were conducted under an approved animal protocol.

This manuscript includes clinical data, which were previously published elsewhere [7–9,25]. The authors declare that all these participants provided written informed consent, and the study design was approved by the Institutional Review Board of the University of Tokyo Hospital. The approval number is 683.

Tumor xenografts in nude mice

Subcutaneous xenograft tumors in BALB/c mice were established by the injection of a 500-μL cell suspension of 10×10^6 AN3CA and HEC-59 endometrial carcinoma cells in PBS. Tumors were removed after exponential growth, cut into 3-mm pieces, and transplanted subcutaneously into other mice. One week after tumor transplantation, mice were assigned randomly to one of three treatment regimens: (1) vehicle (control), (2) NVP-BEZ235, and (3) RAD001. Each treatment group consisted of 6 mice. NVP-BEZ235 and RAD001 were injected orally (p.o.) at daily doses of 40 mg/kg and 2.5 mg/kg, respectively. Tumor volumes (in mm^3) were calculated by the formula: ([major axis]*[minor axis]2/2). After the treatment, the tumors were removed and analyzed by Western blot analysis. Tumor weight (wet weight) was measured, and the average weight was calculated for each group.

Single nucleotide polymorphism typing array and array comparative genomic hybridization

A single nucleotide polymorphism (SNP) array was performed in the HEC-6, 50B, 59, 88, 108, 116, 151, and HHUA cell lines. Experimental procedures for GeneChip were performed according to the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA) with the use of a human mapping 250K Nsp array [9]. Array comparative genomic hybridization (CGH) was performed using arrays of 2464 BAC clones (HumArray2.0) in the remaining 5 cell lines (AN3CA, HEC-1B, Ishikawa, KLE, and RL95-2) as described previously [7].