High Prevalence of Multiple Human Papillomavirus Infection in Japanese Patients with Invasive Uterine Cervical Cancer

Hidemichi Watari[a]*, Rie Michimata[b,c]*, Motoaki Yasuda[d]*, Akihiro Ishizu[e],
Utano Tomaru[b], Ying Xiong[a], Mohamed K Hassan[a], Noriaki Sakuragi[a]

[a]Department of Obstetrics and Gynecology, Hokkaido University Graduate School of Medicine, Sapporo, Japan.
[b]Department of Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Japan.
[c]GeneticLab, Co., Ltd., Sapporo, Japan.
[d]Department of Oral Pathobiology, Hokkaido University Graduate School of Dental Medicine, Sapporo, Japan.
[e]Faculty of Health Sciences, Hokkaido University, Sapporo, Japan.

To whom correspondence should be addressed: Akihiro Ishizu, MD, PhD, Faculty of Health Sciences, Hokkaido University, Kita-12, Nishi-5, Kita-ku, Sapporo, Japan.
e-mail: aishizu@med.hokudai.ac.jp, TEL: +81-11-706-3385, FAX: +81-11-706-4916

*These three authors equally contributed to this work.

Running title: Multiple HPV infection in invasive cervical cancer

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Abstract

Objective: Multiple human papillomavirus (HPV) infection of the uterine cervix has been suggested as a risk factor for persistent HPV infection, resulting in the development of invasive cervical cancer. The aim of this study was to reveal the actual state of multiple HPV infection in Japanese patients with invasive cervical cancer. ***Methods:*** Freshly-frozen 60 invasive cervical cancer tissues were examined for genotyping of HPV. The presence of HPV genotypes in the cancer tissues was determined by an HPV-DNA array, which can discriminate 25 different HPV genotypes with high sensitivity and specificity. ***Results:*** Among 60 samples, 59 (96.7%) were positive for HPV. The three common genotypes were HPV-16 (83.3%), HPV-18 (45.0%) and HPV-52 (28.3%). Multiple HPV infection was observed in 47 of 60 samples (78.3%), in which 42 cases were infected with more than one high-risk genotypes (70.0%). The multiple high-risk HPV infection was significantly more prevalent in patients below 40 years old (14/15, 93.3%) than in patients 40 years and over (28/45, 62.2%). ***Conclusion:*** The HPV-DNA array is a preferable method to detect HPV genotypes. Multiple HPV infection in Japanese patients with invasive cervical cancer seemed to be more frequent than reported in the literature.

Introduction

Uterine cervical cancer is the second most common cancer in women worldwide. Certain genotypes of human papillomavirus (HPV) have been shown to be closely associated with the development of cervical cancer. Currently, more than 90 different HPV genotypes have been identified. Genotypes, such as HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -66, are regarded as high-risk types because they are identified in high-grade squamous intraepithelial lesions (HSILs) and invasive cervical cancer tissues [1, 2]. On the other hand, the genotypes HPV-6 and HPV-11 are considered as low-risk types [2]. Today, HPV genotyping information is clinically useful for prediction of prognosis and therapy based on the risk [3].

The frequency of HPV genotypes detected in cervical cancer tissues varies according to several factors, including the method of HPV detection and the ethnicity. More sensitive and reliable methods rather than individual testing such as polymerase chain reaction (PCR), restriction fragment length polymorphism, and Southern blot, should be developed as screening tools for HPV detection. Conventional methods of individual detection of each genotype have been replaced by a high-throughput technology, which can identify many HPV genotypes at once. In the present study, a DNA array-based method to detect 25 different HPV genotypes, containing the 12 high-risk types, was applied in order to detect multiple HPV infection. A recent study revealed that multiple HPV infection is a factor implicated in persistent HPV infection [4]. Some studies also indicated that persistent HPV infection is critically implicated in the development of cervical dysplasia [4, 5]. Collectively, multiple HPV infection is likely to be associated with the development of cervical neoplasm. However, the prevalence of multiple HPV infection in invasive cervical cancer in Japan has not been elucidated. An important factor that can affect HPV genotyping results is the quality of

the template DNA. Fresh specimens can yield DNA with better quality than formalin-fixed and paraffin-embedded tissues for amplification. Therefore, this study used freshly-frozen tissue specimens from consecutive patients newly diagnosed as invasive cervical cancer and applied an HPV-DNA array to clarify the status of multiple HPV infection.

Materials and Methods

Patients

A total of 60 invasive cervical cancers were obtained from archives of freshly-frozen tissues between the period of 1999 and 2004 at the Department of Gynecology of Hokkaido University, Sapporo, Japan. Forty-nine surgical specimens were obtained by radical hysterectomy. Eleven biopsy specimens were taken from patients who received radiotherapy and/or platinum-based chemotherapy as the initial treatment. In both cases, 5 mm cubic tissues were obtained from the inside of the tumors to avoid contamination of the extrinsic HPV. The age of patients ranged from 20 to 72 years old with an average of 48.5 years (Table 1). An oncologic gynecologist performed pelvic examination and staging of the women according to the FIGO classification. Based on histological examinations, the tumors were divided into squamous cell carcinoma (Sq; 42 cases, 70.0%) and non-squamous cell cacrinoma (Non-Sq; 18 cases, 30.0%). Non-Sq consisted of 13 endocervical adenocarcinomas, 3 adenosquamous carcinomas, and 2 small cell carcinomas. The status of lymph node metastasis was recorded from 49 patients who undertook radical hysterectomy.

HPV-DNA array

The HPV-DNA array used in this study was designed to detect 12 high-risk HPVs (HPV-16/18/31/33/35/39/45/51/52/56/58/59), 2 intermediate-risk HPVs (HPV-30/53), 7 low-risk HPVs (HPV-6/11/34/40/42/54/61), as well as 4 skin-type HPVs (HPV-17/20/21/47). The target sequences of these 25 genotypes and the G3PDH gene were amplified from the plasmids containing the genes by PCR using primer sets listed in Supplementary Table 1. Each 4 ng of the amplified HPV-DNA and G3PDH sequence was purified using a spin column, and then blotted onto Hybond-N+ membrane (Amersham Biosciences, Tokyo, Japan). The schema of the HPV-DNA array is shown as Supplementary Figure 1.

Genomic DNA was extracted from freshly-frozen tissues of 60 invasive cervical cancers using PureGene DNA isolation kit (QIAGEN, Tokyo, Japan) and stored at -20°C until use. For probe labeling, genomic DNA templates (100 ng) were amplified, using 26 sets of primers (including the primer sets for 25 HPV genotypes and G3PDH, final 0.1 mM each, Supplementary Table 1) and biotinylated dUTP (Roche Diagnostic, Tokyo, Japan). The cycle sequence was as follows; denaturalization at 95°C for 20 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds (25 cycles) followed by incubation at 72°C for 5 minutes. The labeled probes were purified using a spin column and stored at -20°C until use. The array membrane was pre-hybridized with PerfectHyb solution (Toyobo, Osaka, Japan) for 1 hour at 72°C. Then, the labeled probes (biotin-incorporated PCR products) were denatured, and then hybridized with the membrane. Hybridization was done at 72°C for more than 12 hours followed by highly stringent washes at 72°C. Signal detection was performed using Phototope-Star Detection kit for nucleic acids (New England Biolabs, Ipswich, MA) and CDP-star (Roche Diagnostic) according to the manufactures' protocols. Finally, chemi-luminescent signals were measured using VersaDoc3000 system (BioRad Laboratories, Tokyo, Japan).

Statistical analysis

The correlation between the status of HPV infection and the clinicopathological factors, including age, clinical stage, histology, and lymph node metastasis, was examined. For statistical analysis, the Chi-square test or Fisher's exact test was used. P values less than 0.05 were considered to be significant. Statistical analyses were performed with Statview software (SAS Institute, Cary, NC).

Results

Specificity of the HPV-DNA array

To evaluate the specificity of the HPV-DNA array, the plasmid containing each sequence of HPV genotype (HPV-6/11/16/17/18/20/21/30/31/33/34/35/39/40/42/45/47/51/52/53/54/56/58/59/61) or G3PDH was amplified by multiplex PCR, using 26 sets of primers listed in Supplementary Table 1. When the labeled probes were hybridized with the array membranes, most primer sets were specific for the corresponding template DNA, though a few cross hybridization was seen between HPV-20 and HPV-21 (Figure 1). Although further improvement is needed to dissolve the cross hybridization, we applied the HPV-DNA array for this study because HPV-20 and HPV-21 are skin-type HPVs.

Genotypes detected in invasive cervical cancer tissues by the HPV-DNA array

The representative results of HPV detection in cervical cancer tissues by the HPV-DNA array are shown in Figure 2. Data were considered to be valid only when the positive control signals of G3PDH were seen. All positive signals were validated by

direct sequencing (data not shown). Table 2 summarizes the distribution of HPV genotypes according to the histology. HPV was positive in 59 of 60 cases (98.3%). Among high-risk types, HPV-16, -18, -31, -33, -35, -52, and -58 were detected. The most prevalent genotype was HPV-16 (50/60, 83.3%), followed by HPV-18 (27/60, 45.0%) and HPV-52 (17/60, 28.3%). The prevalence of HPV-18 was significantly more frequent in Non-Sq (14/18, 77.8%) than in Sq (13/42, 31.0%) (p=0.0008). HPV-52 and HPV-58 were observed exclusively in Sq. Among the low-risk types, HPV-11 (17/60, 28.3%) and HPV-34 (8/60, 13.3%) were detected.

Correlation between multiple high-risk HPV infection and clinicopathological factors

In the present study, 47 of 60 patients (78.3%) showed multiple HPV infection, in which the rate was extremely higher than the rates described previously (Table 3, see references [6-23]). Among the 47 patients, multiple high-risk HPV infection was observed in 42 patients (42/60, 70.0%, Table 4). To get a better understanding of the clinical significance of multiple high-risk HPV infection in invasive cervical cancer, the correlation between the status of multiple high-risk HPV infection and various clinicopathological factors was examined. Multiple high-risk HPV infection was observed in 14 of 15 (93.3%) patients below 40 years old, and in 28 of 45 (62.2%) patients 40 years and over. The difference in positive rate was statistically significant (p=0.03). Multiple high-risk HPV infection was not associated with the clinical stage (Ib/II versus III/IV, p=0.95), histology (Sq versus Non-Sq, p=0.71), and status of lymph node metastasis (Negative versus Positive, p=0.73).

Discussion

HPV infection is considered a major causal factor of anogenital malignant lesions. Currently, more than 90 different HPV genotypes have been identified. The difficulty in HPV genotyping is brought by the sequence homology of viral genome, which will cause the cross hybridization in Southern blot technique or unspecific amplification in PCR method. Although DNA sequencing technology is able to distinguish each HPV genome sequence, it will require a lot of time and laboratory effort. Comprehensive HPV detection systems, including a DNA array-based detection technology, may solve these problems. The HPV-DNA array system used in this study is a specific method to detect 25 different HPV genotypes without any significant cross hybridization (Figure 1).

The International Agency for Research on Cancer reported that the top 5 prevalent HPV genotypes in invasive cervical cancer were HPV-16 (53%), HPV-18 (15%), HPV-45 (9%), HPV-31 (6%), and HPV-33 (3%) [24]. In this study, the top 5 prevalent HPV genotypes were HPV-16 (83.3%), HPV-18 (45.0%), HPV-52 (28.3%), HPV-31 (15.0%), and HPV-58 (13.3%). The high prevalence of HPV-16 and HPV-18 in Japanese patients with invasive cervical cancer is a similar finding to that in the world. The prevalence of HPV-18 was even so high in this study. This may be attributed to the high proportion of adenocarcinoma in our patients because HPV-18 is closely associated with cervical adenocarcinoma rather than Sq [24]. Correspondingly, the prevalence of HPV-18 was significantly more frequent in Non-Sq (14/18, 77.8%) than in Sq (13/42, 31.0%) in the present study. On the other hand, the high prevalence of HPV-52 and HPV-58 in invasive Sq was a common signature in East Asia, including China, Hong Kong, Korea, and Japan, compared to Western countries [24].

The most important issue in this study is that the prevalence of multiple HPV

infection in Japanese patients with invasive cervical cancer is extremely high (47/60, 78.3%). The reported incidence of multiple HPV infection in invasive cervical cancer varies greatly and ranges between 1.5 to 39.6% [6-23]. This result is, at least in part, due to the high sensitivity of the HPV-DNA array system and probably to the good quality of the template DNA used in this study. The DNA array system used in this study can detect 50 fg of the HPV genomic DNA; therefore, it can detect the signal if more than 5 copies of the genotype virus are present (Supplementary Figure 2). In addition, freshly-frozen samples were employed in this study, though other researchers mostly used formalin-fixed and paraffin-embedded tissues. It is well known that the duration of fixation and the type of fixative used affects the quality of the extracted nucleic acids. Degradation of DNA is the most common type of damage. Although a comparative study is needed to determine whether the frequency of multiple HPV infection in invasive cervical cancer in Japan is higher than that in other countries, our data suggest that the “true” positive rate of multiple HPV infection in invasive cervical cancer might be higher than that expected from previous reports.

Clinical significance of multiple HPV infection remains to be elucidated. Ho et al. investigated the natural history of cervicovaginal HPVs, and defined the association between the presence of multiple HPV genotypes and the persistence (over 6 months) of infection [4]. On the contrary, other studies showed that persistent HPV infection was independent to co-infection with other HPV genotypes [25, 26]. The carcinogenic risk of multiple HPV infection is also controversial among reports. Lee et al. reported that patients infected with multiple HPV genotypes had a 31.8-fold higher risk of cervical cancer, while those infected with a single HPV genotype had a 19.9-fold increased risk [27]. On the other hand, Sasagawa et al. reported that the odds ratio of multiple HPV infection was 24 for low-grade squamous intraepithelial lesions, 16 for HSILs, and 8.3 for Sq of the cervix [8]. The correct answer to the question about the

association between multiple HPV infection and the pathogenesis of the disease may have to await further large-scale studies.

In conclusion, our study demonstrated the high frequency of multiple HPV infection in Japanese patients with invasive cervical cancer, especially women below 40 years old. It would be worth establishing whether the presence of multiple HPV infection in uterine cervix would be a marker for persistent HPV infection and progression of HPV-related diseases in the future.

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Table 1. Clinicopathological characteristics of patients enrolled in this study

Factors	n (%)
Age	60 (100)
-29	3 (5.0)
30-39	12 (20.0)
40-49	15 (25.0)
50-59	20 (33.3)
60-	10 (16.7)
Clinical stage	60 (100)
Ib	28 (46.6)
IIa	2 (3.4)
IIb	15 (25.0)
IIIa	1 (1.7)
IIIb	10 (16.7)
IVa	3 (5.0)
IVb	1 (1.7)
Histology	60 (100)
Squamous cell carcinoma (Sq)	42 (70.0)
Non-squamous cell carcinoma (Non-Sq)	18 (30.0)
Lymph node metastasis	49 (100)
Negative	34 (63.4)
Positive	15 (36.6)

Table 2. Distribution of HPV genotypes detected in 60 invasive cervical cancers

HPV genotype	Histology		n (%)
	Sq (/42)	Non-Sq (/18)	
High-risk type			
16	37	13	50 (83.3)
18	13	14	27 (45.0)
31	7	2	9 (15.0)
33	1	1	2 (3.3)
35	1	0	1 (1.7)
52	17	0	17 (28.3)
58	8	0	8 (13.3)
Low-risk type			
11	13	4	17 (28.3)
34	7	1	8 (13.3)

Table 3. Frequency of multiple HPV infection in invasive cervical cancer

Authors	Multiple infection (%)	Reference
Kleter et al.	8/180 (4.4)	[6]
Rolon et al.	21/113 (18.6)	[7]
Sasagawa et al.	10/84 (11.9)	[8]
Schwarz et al.	36/362 (9.9)	[9]
Bachtiary et al.	42/106 (39.6)	[10]
Schellenkens et al.	10/74 (13.5)	[11]
Matsukura et al.	29/250 (11.6)	[12]
Huang et al.	44/152 (28.9)	[13]
Huang et al.	48/149 (32.2)	[14]
Kim et al.	7/98 (7.1)	[15]
Stevens et al.	10/116 (5.2)	[16]
Lai et al.	370/2046 (18.0)	[17]
Vermeulen et al.	28/157 (17.8)	[18]
Pretet et al.	115/516 (22.0)	[19]
Siriaunkgul et al.	21/96 (21.9)	[20]
Munagala et al.	7/40 (17.5)	[21]
Oh et al.	34/742 (4.6)	[22]
Mariani et al.	2/134 (1.5)	[23]
Watari et al.	47/60 (78.3)	Present study

Table 4. Prevalence of multiple high-risk HPV infection in invasive cervical cancers

Factors	Infection status		p-value
	Negative/Single	Multiple	
Age			0.03
-39	1	14	
40-	17	28	
Clinical stage			0.95
Ib/II	12	33	
III/IV	6	9	
Histology			0.71
Sq	12	30	
Non-Sq	6	12	
Lymph node metastasis			0.73
Negative	9	25	
Positive	3	12	

Figure legends

Figure 1. Specificity of the HPV-DNA array.

The plasmids containing the following sequence were used as templates; a: HPV-6, b: HPV-11, c: HPV-16, d: HPV-17, e: HPV-18, f: HPV-20, g: HPV-21, h: HPV-30, i: HPV-31, j: HPV-33, k: HPV-34, l: HPV-35, m: HPV-39, n: HPV-40, o: HPV-42, p: HPV-45, q: HPV-47, r: HPV-51, s: HPV-52, t: HPV-53, u: HPV-54, v: HPV-56, w: HPV-58, x: HPV-59, y: HPV-61, and z: G3PDH. The templates were amplified, using the 26 sets of primers (including the primer sets for the 25 HPV genotypes and G3PDH) and biotinylated dUTP. The labeled probes were hybridized with the array membranes, and then signals were detected as described in the Materials and Methods section. Most primer sets were specific for the corresponding template DNA, though a few cross hybridization was seen between HPV-20 and HPV-21.

Figure 2. Representative results of HPV detection by the HPV-DNA array.

A, B: Examples of single infection (A: HPV-16, B: HPV-18). C, D: Examples of double infection (C: HPV-16/18, D: HPV-16/31), E: Example of triple infection (HPV-16/18/52), F: Example of quintuple infection (HPV-11/16/18/31/34).

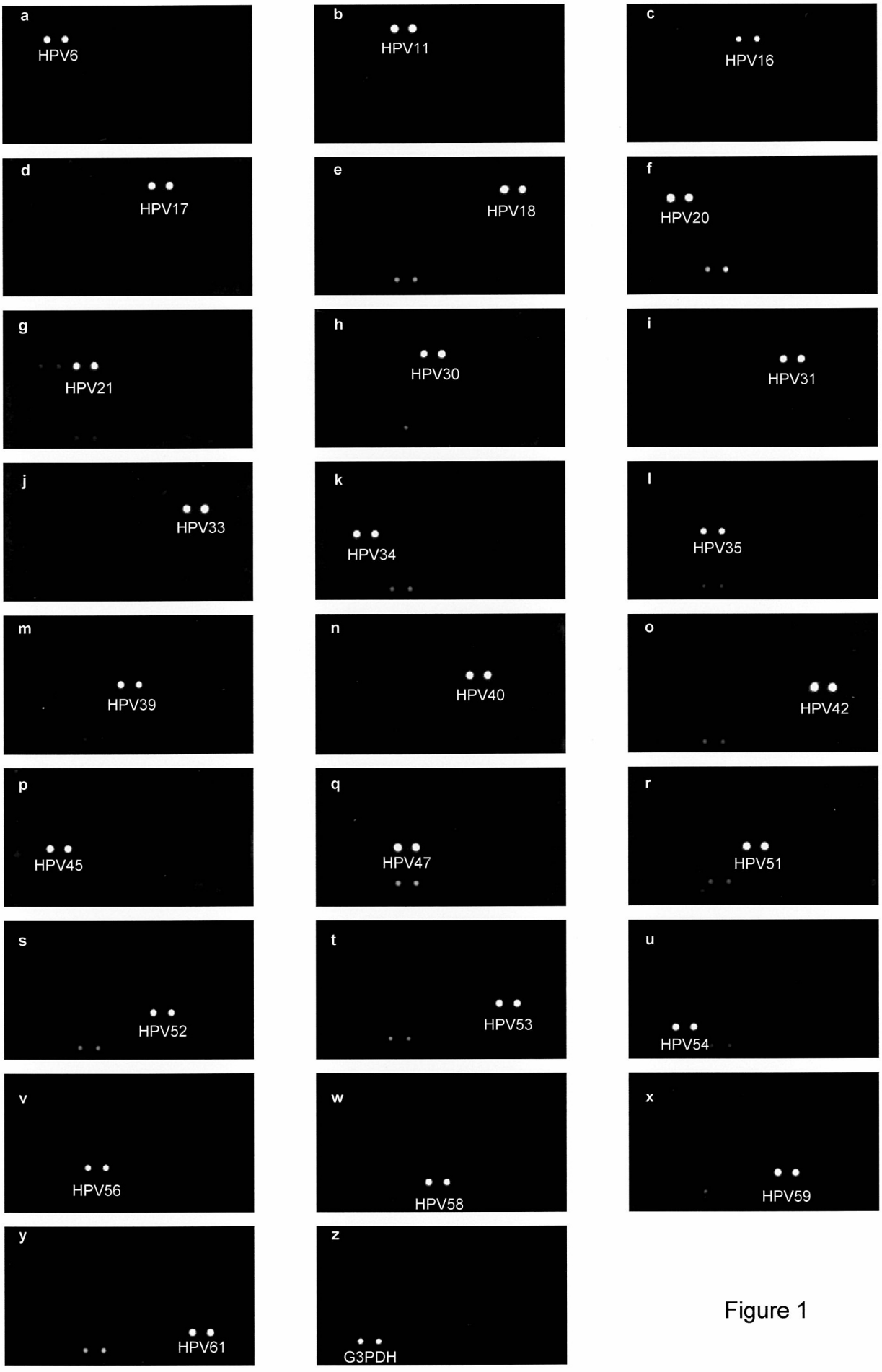
Supplementary Figure 1. Schematic presentation of the HPV-DNA array.

Each membrane contains duplicate spots in which the target sequences of the 25 HPV genotypes and G3PDH gene are blotted. The G3PDH gene is the internal control for amplification.

Supplementary Figure 2. Sensitivity of the HPV-DNA array.

To determine the sensitivity of the HPV-DNA array system, the following amount of

templates were applied for probe labeling; a: HPV-16 10 pg , b: HPV-16 100 fg , c: HPV-16 10 fg , d: HPV-58 10 pg , e: HPV-58 100 fg , f: HPV-58 10 fg , g: HPV-11 1 ng + HPV-16 1 ng + HPV-18 1 ng + HPV-52 1 ng + HPV-59 1 ng, h: HPV-11 1 ng + HPV-16 1 ng + HPV-18 50 fg + HPV-52 50 fg + HPV-59 100 fg , i: HPV-11 100 fg + HPV-16 1 ng + HPV-18 100 fg + HPV-52 50 fg + HPV-59 50 fg, followed by hybridization with the array membrane. Even when multiple genotypes were present in the templates, signals could be detected if the amount of DNA from each genotype reached 50 fg.


a
HPV6
b
HPV11
c
HPV16
d
HPV17
e
HPV18
f
HPV20
g
HPV21
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HPV30
i
HPV31
j
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HPV34
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HPV35
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HPV39
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HPV42
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HPV45
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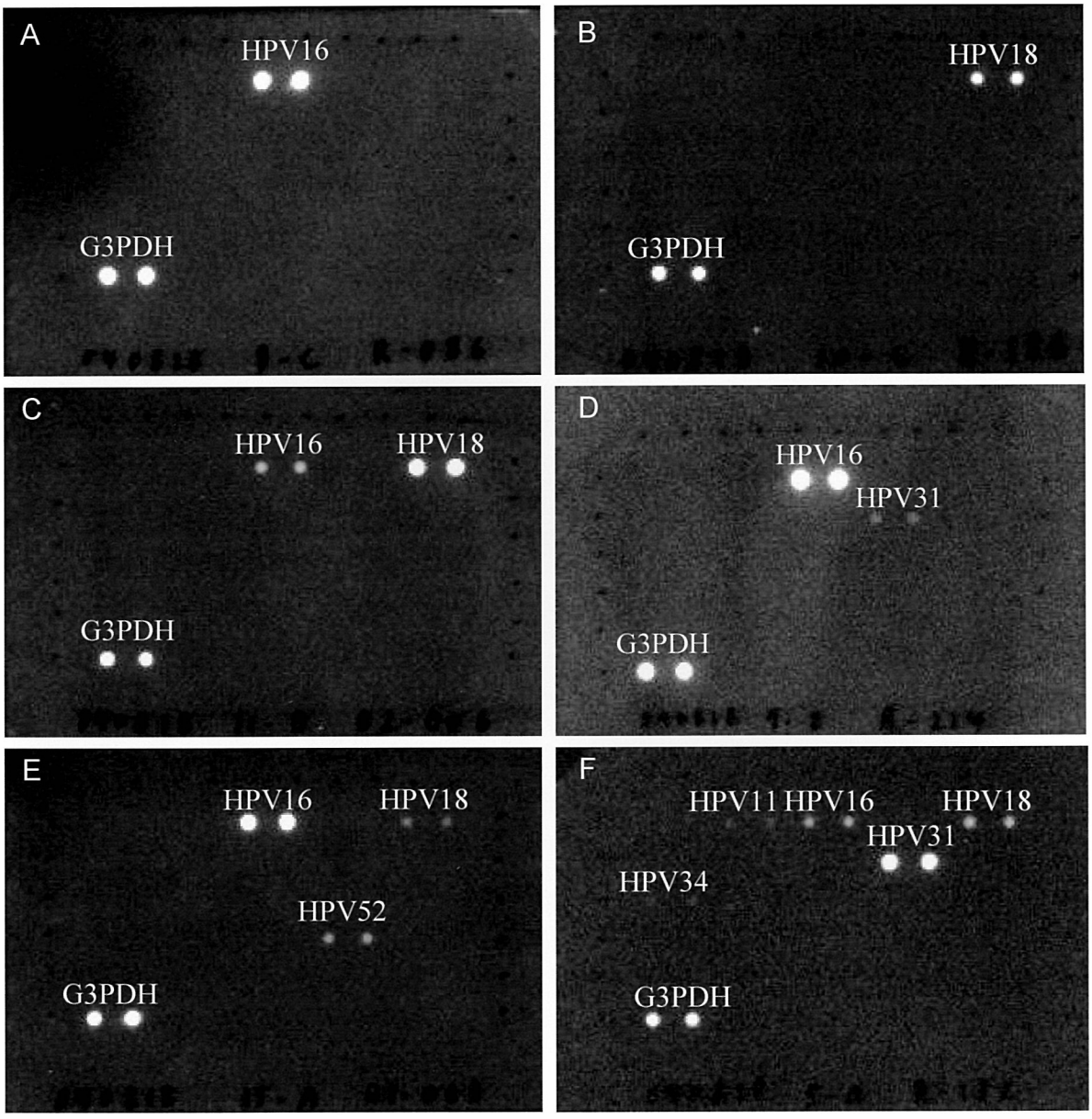
HPV59
y
HPV61
z
G3PDH

Figure 1


A
HPV16
G3PDH
B
HPV18
G3PDH
C
HPV16
HPV18
G3PDH
D
HPV16
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G3PDH
E
HPV16
HPV18
HPV52
G3PDH
F
HPV11
HPV16
HPV18
HPV31
HPV34
G3PDH

Figure 2