A Large Case-Control Study of Cervical Cancer Risk Associated with Human Papillomavirus Infection in Japan, by Nucleotide Sequencing–Based Genotyping

Tsuyoshi Asato,[1] Toshiyuki Maehama,[2] Yutaka Nagai,[2] Koji Kanazawa,[2] Hiroshi Uezato,[3] and Ken-ichi Kariya[1]

[1]Division of Cell Biology, Graduate School of Medicine, [2]Department of Obstetrics and Gynecology, Faculty of Medicine, and [3]Department of Dermatology, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan

Using nucleotide sequencing–based genotyping, we conducted a case-control study to examine cervical cancer risk associated with human papillomavirus (HPV) infection in a Japanese population. A consensus primer pair was used to amplify DNA from the L1 region of HPV by polymerase chain reaction (PCR). By PCR, 311 of 356 patients with cervical cancer and 333 of 3249 control individuals were positive for HPV. By the direct sequencing of PCR products, nucleotide sequences of 30 genotypes were obtained. A high incidence of type 52 and a low incidence of type 16 were characteristic of the control group. Odds ratios were estimated for 18 genotypes. Types 71, 90, and 91, previously uncharacterized, were classified as low-risk genotypes, which is consistent with predictions made on the basis of phylogeny. The present study is the first large case-control study of its kind to use nucleotide sequencing–based genotyping.

Human papillomavirus (HPV) is the major causative factor in cervical cancer [1]. To date, >90 HPV genotypes have been reported. Among them, types 16 and 18 are commonly detected in patients with cervical cancer and, therefore, have been classified as high-risk genotypes [2–4]. However, the risks associated with uncommon genotypes have not yet been definitely established. One reason for this is that methodologies currently employed in epidemiological studies have limited ability to detect HPV. In the majority of current studies, the L1 region of HPV DNA is amplified by polymerase chain reaction (PCR) with consensus primers [5], and the PCR products are subjected to further analysis by hybridization with genotype-specific probes [5]. Our previous method was to first test for the presence of HPV by L1-primer PCR and then to distinguish HPV genotypes by E1-primer PCR. The L1 primers were designed by Yoshikawa et al. [6], who have described their own PCR-based approach (PCR followed by a restriction enzyme fragment polymorphism analysis); the E1 primers were designed by us [7–9]. However, none of these methodologies can detect all genotypes [5–9]. To overcome this limitation, we decided to determine the nucleotide sequences of all L1 PCR products; in principle, this approach can detect all genotypes. Another reason for the lack of information about the cervical cancer risk associated with uncommon HPV genotypes is that previous case-control studies have included insufficient numbers of control individuals. Even the largest pooled study conducted to date [3] included <2000 control individuals. Here we report the results of the first large case-control study of its kind that uses nucleotide sequencing–based genotyping and that includes >3000 control individuals. The present study provides, for the first time, information on the cervical cancer risks associated with a number of HPV genotypes.

Patients, materials, and methods. All study participants were residents of Okinawa prefecture, which lies apart from the main islands of Japan and has a population of 1.35 million people. In Okinawa, Ryukyu University Hospital operates a prefecture-wide cervical cancer registry and treats >90% of the patients living in the prefecture who have invasive cervical cancer.

Cervical swab samples were obtained from women who consulted Ryukyu University Hospital during 1993–2000. Consent was obtained for testing for HPV. To test for HPV, the cervical swab samples were subjected to DNA extraction, and the DNA was subjected to PCR using an L1 consensus primer pair (L1C1 and L1C2; reported by Yoshikawa et al. [6]), as described elsewhere [7]. PCR with a β-globin primer pair was performed in parallel, and β-globin–negative samples were not included in further analyses. The L1 PCR products obtained from HPV-positive samples were stored frozen. The present study included only previously untreated patients who subsequently received histological diagnoses of invasive squamous cell carcinoma. Of 356 such patients with cancer (age range, 21–93 years; average age, 53.4 years [≤29 years, 3.7%; 30–39 years, 18.8%; 40–49 years, 21.1%; 50–59 years, 20.5%; 60–69 years, 20.2%; 70–79

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Reprints or correspondence: Dr. Ken-ichi Kariya, Div. of Cell Biology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara-cho, Okinawa 903-0215, Japan (kariya@med.u-ryukyu.ac.jp).

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(16型) 16人 / 3249人 = 0.492% ≒ 0.5%

(18型) 6人 / 3249人 = 0.1846% ≒ 0.2%

years, 9.8%; ≥80 years, 5.9%]), there were 311 (87.4%; age range, 24–89 years; average age, 54.4 years) who were positive for HPV.

Control individuals included both women who had consulted Ryukyu University Hospital and the participants in cervical cancer screenings conducted in Okinawa during 1993–1995. The screenings were held in a number of locations so that women from most regions of the prefecture could participate. Cervical swab samples were subjected to HPV testing as described above, with the approval of city governments and of the Okinawa General Health Service Association, which conducted the screenings. The association provided us only with information on ages and cytology of the participating women. Women with no cytological abnormalities (Papanicolaou class I or II, with no koilocytosis) were regarded as control individuals, and their L1 PCR products were stored frozen. Of 3249 control individuals (age range, 18–85 years; average age, 52.4 years [≤29 years, 5.1%; 30–39 years, 14.2%; 40–49 years, 20.0%; 50–59 years, 25.9%; 60–69 years, 25.6%; 70–79 years, 8.9%; ≥80 years, 0.4%]), there were 333 (10.2%; age range, 18–85 years; average age, 50.7 years) who were positive for HPV.

For nucleotide sequencing, stored L1 PCR products were thawed and subjected to electrophoresis on 2.5% agarose gel containing ethidium bromide. The bands (~250–260 bp) were excised from the gel, and DNA was extracted and purified by use of the QIAquick Gel Extraction Kit (Qiagen). To prepare the sequencing templates, purified DNA was subjected to a second PCR, with the following modified consensus primers: L1C1B (5′-CCCGGGATCCCGTAAACGTTTTCCCTATTTT-TTT-3′) and L1C2B (5′-CCCGCTGCAGTACCCTAAATACTC-TGTATTG-3′). These primers contained original L1C1 and L1C2 primer sequences plus an additional 10 nt (indicated above by underscoring), to facilitate direct sequencing. This secondary PCR reaction (100 μL) contained ~20 ng of template DNA, 50 pmol of each primer, 200 μmol of each dNTP/L, and 2 U of ExTaq polymerase (TaKaRa). Thermal-cycling conditions were as follows: initial denaturation at 94°C for 2 min; 20 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 30 s, and extension at 70°C for 1 min; and final extension at 70°C for 5 min. The PCR products were gel purified as described above.

For sequencing reactions by use of a BigDye Terminator Kit (Applied Biosystems), ~5 ng of the gel-purified secondary PCR products were used as templates. The original L1C1 and L1C2 primers were used for the sequencing of the templates directly on both strands. With the aid of the 10 terminal base pairs that had been added to the templates by the secondary PCR, these primers annealed stably to the templates and performed well in sequence reactions. Sequences of the region between the 2 primers were obtained by use of the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The similarity between the obtained L1 sequences and those of various HPV genotypes in the GenBank database was determined by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST).

The χ^2 test was used to compare the prevalence of different genotypes. Odds ratios (ORs) and 95% confidence intervals (CIs) were used to assess the cervical cancer risks associated with individual genotypes.

Table 1. Prevalence of and cervical cancer risks associated with human papillomavirus (HPV) genotypes, detected by nucleotide sequencing.

HPV genotype	No. (%) of study participants positive for HPV: Patients with cervical cancer (n = 311)	Control individuals (n = 333)	OR (95% CI)[a]
6	0 (0.0)	3 (0.9)	...
16	132 (42.4)	16 (4.8)	534.6 (294.4–970.8)
18	24 (7.7)	6 (1.8)	259.2 (101.1–664.8)
31	19 (6.1)	9 (2.7)	136.8 (58.7–318.8)
32	0 (0.0)	1 (0.3)	...
33	28 (9.0)	12 (3.6)	151.2 (72.3–316.1)
35	13 (4.2)	27 (8.1)	31.2 (15.1–64.4)
39	0 (0.0)	3 (0.9)	...
42	0 (0.0)	6 (1.8)	...
45	1 (0.3)	0 (0.0)	...
51	4 (1.3)	28 (8.4)	9.3 (3.1–27.5)
52	22 (7.1)	40 (12.0)	35.6 (19.6–64.8)
53	4 (1.3)	19 (5.7)	13.6 (4.5–41.7)
54	1 (0.3)	3 (0.9)	21.6 (2.2–211.7)
56	7 (2.3)	18 (5.4)	25.2 (10.0–63.3)
58	25 (8.0)	9 (2.7)	180.0 (79.5–407.4)
59	4 (1.3)	5 (1.5)	51.8 (13.5–199.4)
61	0 (0.0)	4 (1.2)	...
66	3 (1.0)	3 (0.9)	64.8 (12.7–329.8)
67	0 (0.0)	1 (0.3)	...
68	3 (1.0)	16 (4.8)	12.2 (3.4–43.2)
70	2 (0.6)	4 (1.2)	32.4 (5.8–181.4)
71	0 (0.0)	10 (3.0)	...
72	0 (0.0)	5 (1.5)	...
73	1 (0.3)	0 (0.0)	...
82	1 (0.3)	1 (0.3)	64.8 (4.0–1052.3)
84	0 (0.0)	5 (1.5)	...
86	0 (0.0)	2 (0.6)	...
90[b]	1 (0.3)	12 (3.6)	5.4 (0.7–42.4)
91[b]	1 (0.3)	12 (3.6)	5.4 (0.7–42.4)
ND	15 (4.8)	53 (15.9)	...
Total	311 (100.0)	333 (100.0)	...

NOTE. CI, confidence interval; ND, not determined; OR, odds ratio.

[a] For estimation of ORs and 95% CIs, patients with cervical cancer who were negative for HPV (n = 45) and control individuals who were negative for HPV (n = 2916) were used as reference categories.

[b] L1 regions of these genotypes are not amplified by the MY09/MY11 primers [10].