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Analysis of HPV-other Samples by Performing HPV DNA Sequencing

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Background : HPV-other samples are designated as being positive on HPV-PCR, but negative when using specific HPV hybridization probes. We wanted to determine the types on the HPV-other samples by performing sequencing, and to know the pathologic status of the uterine cervix according to the HPV type detected on sequencing. **Methods :** For HPV genotying, we used the commercially available HPV DNA Chip test, which contains 15 types of high-risk HPV and 9 types of low-risk HPV. The HPV DNA sequencing was performed for the HPV-other samples of 209 patients who subsequently underwent cervical biopsy. **Results :** For 204 of the 209 samples, the HPV types detected by sequencing were absent types at used HPV DNA chip. For the remaining 5 samples, sequencing was impossible due to mixed peaks. HPV-81 (19.6%), HPV-61 (18.6%), HPV-62 (16.7%) and HPV-84 (13.9%) were frequently detected. For the HPV-81, -62, -71, and -72 samples, most of the samples displayed normal or LSIL. However, HPV-84 and -61 were more associated with HSIL or worse, as compared to the other types. **Conclusion :** HPV-81, -61, -62 and -84 were frequently found on sequencing analysis of the HPV-other samples. The pathologic status was diverse, according to the HPV type detected on sequencing.

Key Words : DNA chips; Papillomaviridae; Sequence analysis, Uterine cervix

Infection with specific types of HPV appears to be an essential step in the development of invasive cervical cancer and also its precursor lesion, squamous intraepithelial lesion (SIL).[1,2] To date, More than 85 HPV types have been currently identified, of which at least 35 types have been found in infections of the female genital tract.[3,4] HPVs have been categorized into the "high risk" and "low risk" types based on their relative risks for the occurrence of a high-grade cervical lesion and invasive cancer.[5]

The HPV DNA Chip test has recently been used for evaluating uterine cervical lesions as a diagnostic tool because it easily discriminates between many HPV genotypes and it can also identify multiple viral infections.[6,7] The commercially available HPV DNA chip tests can only detect 20-24 HPV genotypes. Additional HPV types should be added in this test to extend the tsts's clinical usefulness. Therefore, it is necessary to examine the other HPV types (the HPV-other types) that are positive on HPV-PCR, but they show no signal on the DNA Chip scanner. We wanted to determine which HPV types that are not detected on the HPV DNA Chip test by performing sequencing analysis of the HPV-other samples. We also tried to determine the pathologic cervical status of the patient according to each type of HPV, as was detected on sequencing analysis.

MATERIALS AND METHODS

For the HPV genotying, we used a commercially available HPV DNA Chip (MyGene Company, Seoul, Korea), which is a PCR-based DNA microarray system. The HPV DNA chip contains 24 type specific probes for 15 types of high-risk HPV

(HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-53, HPV-56, HPV-58, HPV-59, HPV-66 and HPV-68) and 9 types of low-risk HPV (HPV-6, HPV-11, HPV-34, HPV-40, HPV-42, HPV-43, HPV-44, HPV-54, and HPV-70). Twenty-four type-specific 30-mer oligonucleotide probes containing an amine group at the 5' terminus are immobilized onto a chip slide glass. A slide has eight chambers, and each chamber is used for a test. Briefly, the target HPV DNA was amplified by PCR with using the primers (HPV and β-globin) and conditions provided by the MyGene Company. The amplified DNA was then labeled by a single dye, indocardocyanine-dUTP (MEM Life Science Products, Inc., Boston, MA, USA). The PCR product size of the HPV DNA was 150 base pairs (bp) on the gel electrophoresis. The PCR product was next hybridized onto the chip. Hybridization was performed at 43˚C for 90 min and then the product was washed with 3X SSPE for 5 min and 1X SSPE for 5 min and next it was dried at room temperature. The hybridized signals were visualized with a DNA Chip scanner (Scanarray lite; GSI Lumonics®, Ottawa, Ontario, Canada). The samples that showed a positive band of 150 bp on the gel electrophoresis of the HPV-PCR, but a negative hybridized signal on the DNA Chip scanner were designated as HPV-other samples.

Eight hundred fifty-two HPV-other samples are collected at the Department of Pathology in Chonnam National University Hospital between 2003 and 2005, 852 HPV-other samples were collected. Among 852 HPV-other samples, 209 HPV-other samples were selected for our study. Each sample had been individually obtained from the 209 patients. The cervical pathologic status of the 209 patients has been previously determined by cervical punch biopsy that was examined within 1 month after a HPV DNA chip test. This study was approved by the ethics committee of Chonnam National University Hospital. Written informed consent was obtained from each patient. Sequencing analysis for detecting the specific HPV type was performed on the 209 HPV-other samples. The primed PCR product of each sample was added to the sequencing reaction mixture. Sequencing was performed bidirectionally with the BigDye3 terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) and with using a ABI PRISM 310 Genomic Analyzer (PE Applied Biosystems) at a dispending pressure of 600 mbar with 8-ms open times and 65-s cycle times. The sequencing procedure was carried out by stepwise elongation of the primer strand upon cyclic dispensation of the different deoxynucleotide triphosphates (Amersham Pharmacia Biotech). A CCD camera detected the light output that was caused by nucleotide incorporation. The data was obtained in a graphic format.

The histologic diagnoses of the cervical punch biopsies can be classified as follows: normal, low-grade intraepithelial lesion (LSIL), high-grade intraepithelial lesion (HSIL), squamous cell carcinoma (SCC) and adenocarcinoma (AC). The pathologic slides were re-examined by two gynecologic pathologists working independently, and any diagnostic discrepancy between two pathologists was resolved by the 2 pathologists working in consensus.

We performed statistical analysis using Fisher's exact test between the pathologic status and the types represented by the HPV sequencing analysis. All the p-values represent two-sided statistical tests with statistical significance set at $p<0.05$.

RESULTS

Of 204 of the 209 HPV-other samples, 14 HPV types were found via HPV DNA sequencing analysis. All the types detected on the sequencing analysis were the absent types when using the HPV DNA chip instrument. HPV-81 was frequently detected on the HPV sequencing analysis for the HPV-other samples (41 samples, 19.6%). The next types were HPV-61, HPV-62, and HPV-84. HPV-71 and HPV-72 were detected in more than 10 samples. For the remaining five samples (2.4%), genotyping with using DNA sequencing is impossible, and this was probably due to the samples showing a mixed peak on the sequencing data (Table 1, Fig. 1).

The histologic diagnoses for the 209 HPV-other samples were 150 samples of normal (71.8%), 30 samples of LSIL (14.4%), 25 samples of HSIL (12.0%), 1 sample of SCC (0.5%), and 3 samples of AC (1.4%). The histologic diagnoses are also presented in Table 1 according to the type that was detected on the HPV sequencing analysis. For the samples where HPV-81 was detected on the sequencing analysis, most of the samples were histologically diagnosed as normal or LSIL (40 of 41, 97.6%). For the HPV-62, HPV-71, and HPV-72 samples, most of the samples were normal or LSIL. However, for the HPV-84 and HPV-61 samples, they were more associated with HSIL or worse, as compared to the other HPV types (Table 2).

DISCUSSION

In this study, we found that all the HVP types detected on sequencing analysis were not included in the standard HPV DNA chip test. Our previous study demonstrated the standard HPV

Table 1. The pathologic status according to the HPV type, as detected on sequencing analysis

	Normal (%)	LSIL (%)	HSIL (%)	SCC (%)	AC (%)	Total
HPV81	33 (80.5)	7 (17.1)	0 (0.0)	0 (0.0)	1 (2.4)	41
HPV61	27 (69.2)	2 (5.1)	8 (20.5)	1 (2.6)	1 (2.6)	39
HPV62	28 (80.0)	6 (17.1)	1 (2.9)	0 (0.0)	0 (0.0)	35
HPV84	14 (48.2)	6 (20.7)	8 (27.6)	0 (0.0)	1 (3.4)	29
HPV71	10 (66.7)	4 (26.7)	1 (6.7)	0 (0.0)	0 (0.0)	15
HPV72	9 (81.8)	1 (9.1)	1 (9.1)	0 (0.0)	0 (0.0)	11
HPV82	6 (66.7)	0 (0.0)	3 (33.3)	0 (0.0)	0 (0.0)	9
HPV83	5 (71.4)	1 (14.3)	1 (14.3)	0 (0.0)	0 (0.0)	7
HPV69	4 (80.0)	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	5
HPV32	4 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4
HPV73	2 (66.7)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	3
HPV64	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	3
HPV102	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	2
HPV74	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	1
Sequencing impossible	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5
Total	150 (71.8)	30 (14.4)	25 (12.0)	1 (0.5)	3 (1.4)	209

LSIL, low grade intraepithelial lesion; HSIL, high grade intraepithelial lesion; SCC, squamous cell carcinoma; AC, Adenocarcinoma.

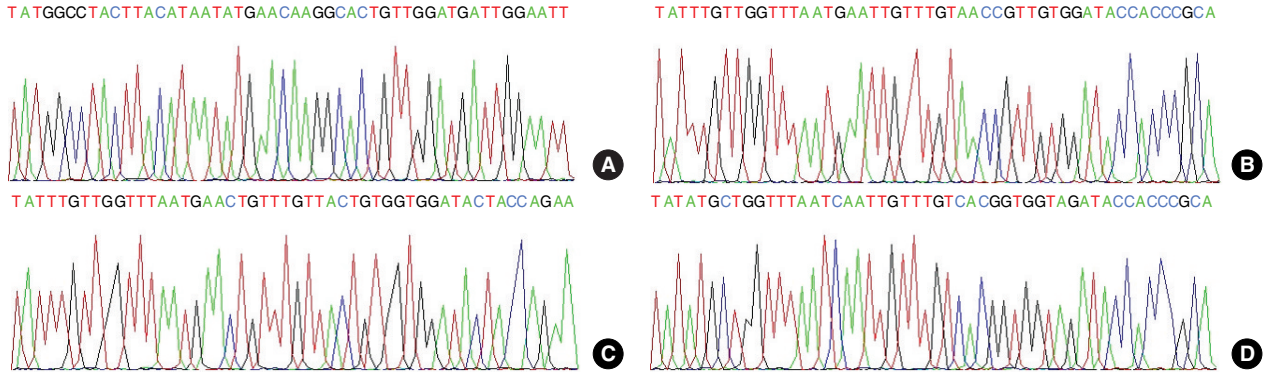

Fig. 1. Examples of the HPV DNA sequencing of the HPV-other samples are noted. (A) HPV-81, (B) HPV-61, (C) HPV-62, (D) HPV-84.

Table 2. The association between pathologic status and HPV types detected on sequencing analysis

HPV types		Pathologic status		p-value
		HSIL or worse	Normal or LSIL	
HPV 84	HPV 84	9	20	0.008
	Non-HPV 84	20	160	
HPV 61	HPV 61	10	29	0.040
	Non-HPV 61	20	150	

HSIL, high grade intraepithelial lesion; LSIL, low grade intraepithelial lesion.

DNA chip test's accuracy for detecting and subtyping the HPV in cervical lesions by comparing the test's results with result of HPV DNA sequencing with using the same samples; we found that the two tests were in agreement in 257 of 282 cases (91.1 %).[8] Therefore, the HPV DNA chip test appears to be an accurate and informative method for evaluating HPV.

The HPV types detected on the HPV sequencing analysis of the HPV-other samples were HPV-81, HPV-61, HPV-62, HPV-84, HPV-71, HPV-72, HPV-82, HPV-83, HPV-69, HPV-32, HPV-73, HPV-64, HPV-102, and HPV-74. As compared with the data of our previous study, the types detected in this study were similar to those of our previous study.[8] Among these types, HPV-81, HPV-61, HPV-62, and HPV-84 were detected at a considerable rate. These types have been recently recognized as new types and they have been categorized into the A3 phylogenetic subgroup.[9] This group was by far the most frequently observed viral HVP group in the patients with an immunosuppressed condition.[10] In one article that was backed by extensive epidemiological data, HPV-64 was a highly prevalent genital papillomavirus that was primarily detected in HIV-infected women.[11] Although the prevalence of the HPV types is different according to race or geography,[12,13] it is necessary that the

A3 group will be included in the commercially used HPV DNA chip test.

On histologic examination, most of the HPV-other samples were normal or LSIL (180/209, 86.1%). However, the histologic status was various according to the types detected on the HPV sequencing. HPV-84 and HPV-61 were more associated with HSIL or worse, as compared to the other HPV-other types. A BLAST homology search demonstrated that HPV-84 is most closely related to HPV 61 (89%), as determined by nucleotide sequence analysis of the L1 open reading frame.[11] Therefore, it is suggested that the behavior of HPV-84 and the behavior of HPV-61 are probably similar.

DNA sequencing is the gold standard method for accurately typing micro-organisms and viruses, and it is more reliable than the hybridization and serological-based techniques, which might be subject to false hybridization and non-specific binding.[14] However, DNA sequencing has limitations for HPV genotyping when the specimen harbors multiple genotypes and the sequencing yields nonspecific amplification products, which causes nonspecific and uninterruptible sequencing data.[15] In this study, HPV genotyping for 5 HPV-other samples was impossible due to the mixed peak in the sequencing data. Baback et al. recently developed type-specific multiple sequencing primers (HPV-6, -11, -16, -18, -31, -33, and -45) to facilitate typing more than one target DNA in the sample.[14,16] If this technique becomes commercially useful, genotyping for the 5 HPV-other samples will probably be done in standard clinical practice.

In summary, HPV-81, HPV-61, HPV-62, and HPV-84 were frequently detected on sequencing analysis of the HPV-other samples. HPV-84 and HPV-61 were comparatively associated with HSIL or even worse. In order to extend the applications of the HPV DNA chip test, additional types should be continuously examined for by performing both sequencing analysis and histologic evaluation.

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