Analysis of human papilloma virus type 52 integration status in exfoliated cervical cells

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Abstract. To explore the significance of human papilloma virus type 52 (HPV52) infection and its integration in cells within cervical lesions, the expression levels of HPV52 were detected using polymerase chain reaction (PCR). The copy numbers of HPV52 E2, HPV52 E6 and the reference gene β-actin were determined by quantitative PCR to analyze the association between HPV52 integration and cervical lesions. HPV52 integration was analyzed by the amplification of papillomavirus oncogene transcripts. A total of 13 samples from 468 cases were positive for HPV52. Among the samples, 1 case with an E2/E6 ratio >1 was purely episomal, 3 cases with an E2/E6 ratio of 0 were purely integrated, and 9 cases with an E2/E6 ratio of between 0 and 1 were a mixture of integrated and episomal. With the progression of cervical disease, the prevalence of the episomal type decreased gradually, and the prevalence of the integrated (episomal and integrated) forms increased. The pure integration of HPV52 occurred in chromosomes 2, 5 and 8. These results indicate that HPV52 integration into the host genome may be a key factor in cervical lesions. Thus, patients at high risk for cervical lesions may potentially be identified by screening for HPV52 infection and integration.

Introduction

Cervical cancer is one of the most commonly occurring cancer types in females worldwide (1-3). Each year, new cases of cervical cancer are detected in ~528,000 women, and the worldwide fatality toll of cervical cancer is ~275,000 (3-5). High-risk human papillomavirus (HPV) infection is the primary cause of cervical carcinogenesis (6). More than 200 different HPV genotypes have been identified, there are >10% differences in the L1 nucleotide sequence between the genotypes (7). A minimum of 13 high-risk HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) (8) are recognized as the causative agents of cervical cancer and numerous other types of cancer (9). HPV16 and HPV18 are the two most prevalent genotypes in cervical cancer (CC) (10). Although the high prevalence of HPV16/18 in CC is common throughout the world, the distribution of other high-risk HPV types in the remaining fraction of CC reveals region-specific variations (11). Notably, in East Asian countries (including Japan, South Korea, Taiwan and China) HPV52 infection is more prevalent compared with European, North American and African regions (12-15). The present study has focused on this type of HPV. The HPV E2 protein controls replication, transcription and viral genome partitioning during the viral infectious life cycle (16). The HPV E2 protein is a key transcriptional regulator of the E6 and E7 genes (17). HPV DNA sequences are typically integrated into the host cell genome, and cervical cancer progression is significantly associated with integration of the viral genome (18-20). Integrated and episomal HPV may be confirmed using a polymerase chain reaction (PCR)-based protocol for the amplification of papillomavirus oncogene transcripts (APOT), which was developed by Klaes et al (21). This protocol is based on the hypothesis that HPV transcripts derived from the integrated HPV genome represent suitable molecular markers for a cervical lesion at risk of progression to carcinoma. However, there are limited available data concerning HPV52 status and integration patterns.

The aim of the present study was to assess HPV52 infection and integration into the host cell genome in exfoliated cervical cells. Briefly, cervical cytology specimens were collected and genomic DNA and RNA were extracted from each sample.
For every specimen HPV DNA was detected using polymerase chain reaction (PCR) amplification with the MY09/11 primers (22), HPV52 was detected using HPV52 type-specific primers. The viral load and integration state were determined for HPV52 positive specimens by quantitative (q)PCR using HPV52 E2, E6 and β-actin primers, the number of copies of HPV52 was calculated using the formula: (E6 copy/β-actin copy) x2, and the HPV52 integration state was determined by the ratio of E2 to E6 copy number. The integration of HPV in the host chromosome integration site may be accurately located by detecting transcription of the poly (A) tail (21,23,24). cDNA was synthesized by reverse transcription using an RNA template, and a (dT)17-p3 as primer; PCR amplification was conducted using cDNA as a template and p1-HPV52 E7 and p3 as primers; nest PCR was conducted using the product as a template and p2-HPV52 E7 and (dT)17-p3 as primers; the PCR product underwent sequence analysis. The integration sites were determined by sequence alignment of the HPV and human chromosome sequence.

Materials and methods

Specimen collection. Cervical cytology specimens from 468 female patients were collected from the Affiliated Hospital of North China University of Science and Technology (Tangshan, China) between October 2012 and June 2014. These specimens were collected through a sterile swab scraped in a clockwise rotation three times in the cervix from every patient, the cervical exfoliation cells were subsequently washed down from the swab using physiological saline solution and collected. The age of the patients ranged from 26-60 years, and the mean age was 41.5±5 years. The patients had no medical history of cervical diseases prior to this diagnosis and were not menstruating or pregnant. The inclusion criteria for all cases was a conventional cervical cytology diagnosis; exclusion criteria were a diagnosis and treatment for cervical intraepithelial neoplasia, cervical cancer and hysterectomy prior to the study. The present study was approved by the Ethics Committee of North China University of Science and Technology, and every specimen donor provided informed consent. These specimens were stored at -80˚C.

Reagents. The Ex Taq Polymerase kit and pMD-18T vector kit were provided by Takara Biotechnology Co., Ltd., Dalian, China. EvaGreen was provided by Biotium, Inc., Freemont, CA, USA. AmpliTaq Gold DNA Polymerase kit was provided by Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA. The RNeasy Mini kit was provided by Qiagen GmbH, Hilden, Germany. Goldview I nuclear staining dye and M-MLV Reverse Transcriptase kit were provided by BioTeke Corporation, Beijing, China.

Media. Dulbecco's modified Eagle medium (DMEM) with antibiotics (100 U/ml penicillin-streptomycin), PBS, fetal bovine serum, trypsin, lysogeny broth and other media used in the present study were provided by BioTeke Corporation.

Plasmid and cell DNA. The plasmid including the complete HPV52 genome, the plasmid encompassing part of the human β-actin gene, 293 and HeLa cell DNA, and E. coli DH5α competent cells, were obtained from the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (Beijing, China).

Cell culture conditions. The HeLa and 293 cells (National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention) were cultivated using 10 ml media (DMEM with 100 U/ml penicillin-streptomycin and 10% fetal bovine serum) in a 10 cm diameter petri dish and maintained at 37˚C in a humidified atmosphere with 5% CO2. The fresh cell media was changed every 2-3 days.

Pathological cytology. The cervical cytology specimens were diagnosed by pathological cytology according to the 2001 Bethesda system (TBS) (25,26). According to the TBS, the cytological diagnosis of abnormal cervical squamous cells is divided into the following categories: Atypical squamous epithelial cells (ASC) of undetermined significance; ASC-cannot exclude high-grade squamous epithelial lesion (HSIL); low-grade squamous intraepithelial lesion (LSIL); and HSIL. Sample smear preparation and staining were performed using BD PrepStain® Slide Processor (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol, and the results were observed using an optical microscope.

Primer design and synthesis. The primers used for HPV52 E2 and E6 were designed according to the HPV52 gene sequence in GenBank (X74481.1; http://www.ncbi.nlm.nih.gov/nuccore/X74481) and were synthesized with the β-actin primer by Sangon Biotech Shanghai Co., Ltd. (Shanghai, China). The primer sequences for HPV52 E2, HPV52 E6 and β-actin are presented in Table I.

Detection of specimen quality. Genomic DNA was extracted from each exfoliated cervical cell sample using the phenol-chloroform method (27,28). The quality of the sample DNA was determined by PCR amplification which was conducted using a Takara Ex Taq kit in a 20 µl reaction mixture [containing 1X Ex Taq Buffer (MgCl2 free), 2.5 mM MgCl2, 0.2 mM dNTPs, 1 unit Ex Taq DNA polymerase, 5 pmol β-actin primers, 40 ng sample DNA template], the 293 cell DNA served as a positive control and sterile water served as a negative control. The thermocycling profile used was as follows: 95˚C for 5 min; followed by 31 cycles of 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec; followed by a final extension at 72˚C for 5 min; and storage at 4˚C.

Detection of HPV DNA. HPV DNA was detected in each exfoliated cervical cell sample using PCR as described above with the MY09/11 primers (MY09: 5'-CGTCCMARRGGAWAC TGATC-3'; MY11: 5'-GCMCAGGGWCATAAYATGG-3'; (amplicon size, 450 bp) for the HPVL1 gene as described previously (22). The PCR products were resolved on a 1% agarose gel and visualized using Goldview I nuclear staining dye. The MY09/11 primers are able to detect >20 HPV types, including HPV52. In the present experiment, HPV infection-free 293 cell DNA was used as a negative control, and HeLa cell DNA and HPV52 plasmid DNA were used as positive controls. HeLa cell DNA was used as a HPV52 type-specific control and HPV52 plasmid DNA was used as a positive control to
detect HPV52 E6 using HPV52 type-specific primers. PCR was conducted in a 20-µl reaction mixture containing 1X Ex Taq Buffer (MgCl₂-free), 2.5 mM MgCl₂, 0.2 mM dNTPs, 1 unit Ex Taq DNA polymerase, 5 pmol of each primer from the primer pair described above (MY09/MY11 or HPV52 E6 primers) and 40 ng sample DNA or control DNA. The thermocycling profile used was as follows: 95℃ for 5 min; followed by 31 cycles of 95℃ for 30 sec, 55℃ for 30 sec and 72℃ for a holding temperature of 4℃. The PCR products were resolved on a 1.0% agarose gel and observed with a UV transilluminator.

**qPCR analysis.** The viral load and integration state were determined using qPCR with HPV52 E2/E6/β-actin primers. Briefly, the number of copies of HPV52 was calculated using the formula: (E6 copy / β-actin copy) x2, and the HPV52 integration state was determined by the ratio of E2 to E6 copy number. The reactions were performed in a 25-µl reaction mixture and standard curves were generated with quantified DNA standards (a 10-fold dilution series of full length HPV52 and β-actin plasmids). The PCR mixtures consisted of 1X AmpliTaq gold buffer (MgCl₂-free), 2.5 mmol/l MgCl₂, 0.2 mmol/l dNTPs, 2 pmol/l primers, 20X EvaGreen (1.25 µl), 0.25 µl RX (stabilizer), 2 µl AmpliTaq Gold DNA polymerase, 0.4 µl DNA template (80 ng/µl) and double distilled water to a final volume of 25 µl. The initial denaturation was performed at 95℃ for 5 min; followed by 5 cycles of 95℃ for 1 min, 55℃ for 1 min and 72℃ for 1 min; followed by 35 cycles at 95℃ for 30 sec, 55℃ for 50 sec and 72℃ for 50 sec; and a final extension at 72℃ for 5 min. The threshold cycle (Cq) (29.30) was determined by the CFX96 Touch Real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), with the baseline set automatically to standard deviations above the background fluorescence generated in the first five cycles. For each specimen, identical amounts of DNA were analyzed for E6 and E2 and compared with expression of the internal reference gene β-actin. The specificity of each amplification was confirmed by checking the dissociation curve against the expected melting temperature of the amplification product.

**Reverse transcription (RT).** RNA was extracted from the patient samples that were identified to have integrated HPV52 using the RNeasy Mini kit. RT was performed using Oligo (dT)₁₇₋₃ (primer: 5’-GACTCGAGTCGACATCGATT  TTTTTTTTTTTTTTTTTTTT; 1 µl), 200 units SuperScript reverse transcriptase (BioTeke Corporation; 1 µl), and total RNA (0.5-1 µg) in a total volume of 20 µl. RNA was reverse transcribed by heating at 42℃ for 50 min, and the reaction was terminated by heating at 70℃ for 15 min. The cDNA samples were stored at 4℃.

To confirm that HPV52 E7 mRNA was detectable in all of the samples and to ensure that the isolated mRNA was of sufficient integrity for PCR amplification, mRNA corresponding to the human reference gene GAPDH was analyzed by RT-PCR. The RT reactions were performed using a Power M-MLV Reverse Transcriptase kit (BioTeke Corporation) according to the manufacturer’s protocol and the cDNA was stored at 4℃. The PCR was conducted using a Takara Ex Taq Polymerase kit in a 20 µl reaction mixture containing 1X Ex buffer (MgCl₂-free), 2.5 mM MgCl₂, 0.2 mM dNTPs, 1 unit Ex Taq DNA polymerase, 5 pmol each GAPDH primer (forward, 5’-CAGTCACCATCGAAMGGA-3’ and reverse, 5’-GTCTAC CACCCATTGCA-3’) and 2 µl cDNA as a template. The thermocycling profile used was as follows: 95℃ for 5 min; followed by 31 cycles of 95℃ for 30 sec, 52℃ for 30 sec and 72℃ for 30 sec; followed by a final extension at 72℃ for 5 min; and storage at 4℃.

### Table I. Primers used in the present study.

| Primer     | Sequence (5’-3’)         | Position | Amplicon size (bp) |
|------------|--------------------------|----------|-------------------|
| HPV52 E2 primers                  | GGAAACGATGGAGTCCGATAC  | 2735-2755 | 176               |
| HPV52 E2-1                            | GTGCGCTATATGGATTTATTCC  | 2891-2910 | 235               |
| HPV52 E2-2                            | CGATGCAAGCAATTTGTG      | 3261-3280 | 245               |
| HPV52 E2-3                            | GTACTTGTGTITGGTGAGTCT    | 3475-3395 |                 |
| HPV52 E2-4                            | GCACCTATAATACACCTAAAGG   | 3604-3626 |                 |
| HPV52 E2-5                            | CACAATGACATGACACCTTG     | 3829-3848 |                 |
| HPV52 E2-6                            | GTTTGAGGATCCAGACAACAC    | 104-123   | 369               |
| HPV52 E6 primers                  | CACCCACACTGTGGCCCATCT    | 453-472   |                 |
| HPV52 E6-1                            | CGCTTGTGTCATTACAATG      | 550-560   | 289               |
| HPV52 E6-2                            | GAAACGCTCATGGCAATG       | 819-838   |                 |

HPV, human papilloma virus.
Detection of viral-cell fusion transcripts using nested PCR. The generation of viral-cellular fusion transcripts encompasses the E6-E7-E1 sequences at their 5'-ends and cellular sequences at their 3'-ends. APOT analysis used an oligo(dT)17-primer coupled to a linker sequence (dT)17-p3, the RT of all of the mRNAs was initiated by binding to their poly(A) tail, and integrate-derived HPV oncogene transcripts were amplified by nested PCR reactions as described previously (21,23,24). The cDNA was synthesized by RT using RNA as a template and (dT)17-p3 as a primer. Nested PCR amplification was then performed. The first PCR reactions were performed using cDNA as a template, and p1-HPV52 E7 and p3 as primers. The second PCR reactions were conducted using first PCR products as template, and p2-HPV52 E7 and (dT)17-p3 as primers. First PCR reactions were conducted with 10 pmol/l primers (forward, p1-HPV52 E7, 5'-CTACCGCTATGCTTATCATAGC-3'; and reverse, p3, 5'-GACTCGAGACATCGCCGCAGG-3'), 1X Ex buffer, 2.5 mmol/l MgCl2, 0.2 mmol/l dNTPs, 1 unit Ex Taq DNA polymerase, and 2 µl cDNA in a total volume of 25 µl; following thermocycling conditions: 95˚C for 5 min; followed by 30 cycles of denaturation at 95˚C for 1 min, annealing at 56˚C for 1 min and elongation at 72˚C for 3 min; followed by a final extension at 72˚C for 5 min. Then, second PCR was performed using the first PCR product (5 µl) as a template, and using the p2-HPV52 E7 forward primer (5'-CGTACTCTCA CAGCAATTGCTGTG-3') and the (dT)17-p3 reverse primer, with the exception that annealing was performed at 67˚C. The positions of the two primers were 751-771 for p1-HPV52 and 787-810 for p2-HPV52. To control for false-positives, a negative control (293 cell DNA template) was included in each set of amplifications. Electrophoresis was performed using a 1.2% agarose gel with Goldview I nuclear staining dye.

Cloning and sequence analysis. To further confirm specific HPV52 oncogene transcription in all of the samples, the final nested PCR products were cloned into a pMD-18T vector according to a previous study (31), except the temperature of the target segment ligation to the pMD-18T vector was changed to room temperature for 1 h. Then Beijing Rui Bo Xing ke Biological Technology (Beijing, China) was commissioned to sequence the products. The results were analyzed using the National Center for Biotechnology Information BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), and HPV52 integration sites in human chromosomes were determined for every integration specimen.

Results

Detection of specimen DNA quality. The 289-bp β-actin product size was confirmed by agarose gel electrophoresis, and PCR amplification of β-actin in the DNA specimens and the positive control was observed to be similar (Fig. 1). The results suggest that all of the DNA samples were of satisfactory quality for subsequent experiments.

Detection of HPV DNA in the samples. In total, 52 HPV-positive samples were identified from among the 468 samples by PCR using the MY09/11 general primer set, which generated a 450-bp product (Fig. 2A). Among them, 13 cases were found to be HPV52-positive using the HPV52 E6 type-specific primer set, which generated a 369-bp product (Fig. 2B).

Association between cervical lesions and HPV52 infection. The 13 HPV52-positive samples were subjected to cytological diagnosis, which indicated that these 13 samples included 1 case of ASC (Fig. 3A), 5 cases of LSIL (Fig. 3B) and 7 cases of HSIL (Fig. 3C).

Analysis of HPV52 infection and viral load. The standard curve and copy number of β-actin, E2 and E6 for every sample were automatically generated and determined by the qPCR instrumentation based on six 10-fold dilutions of β-actin, E2 and E6 standard plasmids. The HPV52 viral load of each sample was calculated (Table II). No clear association of lesion pathology with viral load was observed.

The HPV52 integration status was determined based on the E2/E6 ratio. Among the 13 HPV52-positive samples, 1 case was episomal, and the E2 gene was not destroyed. A total of 3 cases were fully integrated, with E2/E6=0. The remaining 9 cases were a mix of integrated and episomal, with an E2/E6 ratio >0 and <1. The results are presented in Table II. As the pathology of the lesions advanced from ACS to LSIL and HSIL, the proportion of episomal cases decreased and that of integrated (episomal + integrated) cases increased. These results indicate that the pathology of the cervical lesions is associated with HPV52 infection and viral DNA integration into the host genome.

Location of HPV52 integration in the host chromosome. RNA was extracted from the HPV52-integrated samples.
A product of ~600 bp was generated according to nested PCR using the E7-specific primers with the template cDNA obtained by RT with oligo (dT)_{17}-p3. In 3 samples, the HPV52 integration site was in human chromosome 2, 5 or 8; this was determined by sequencing the ~600 bp product and cloning it into the pMD-18 vector. The sequencing results indicated that the HPV52 E7 PCR product that was amplified using the p2-HPV52 primer was part of the HPV52 E7-E1 sequence (Fig. 4). Another section of the amplified sequence was similar to human chromosome 2 (Fig. 5A), 5 (Fig. 5B) or 8 (Fig. 5C).

Discussion

Persistent high-risk HPV infection, with HPV types including HPV16, 18 and 31, has been identified as the major causative agent of cervical cancer (32). HPV52 infection is an important factor for cervical, anal and oropharyngeal carcinoma (33-36). Viral load is recommended as a precancer biomarker, but this has been indicated only for HPV16 infection (37,38). qPCR is regarded as the standard method for viral load quantification (39). Although viral load has been recommended as a precancer biomarker for HPV16 infection (37-39), there is no consistent evidence that viral load is a useful marker of prevalent disease or disease progression (40-42).

In the present study, the results revealed that viral load did not have a clear association with cervical lesion pathology. Multiple E1-L1/E6E7 (including E1/E6E7 and L1/E6E7) ratio analysis has been demonstrated to be more sensitive than E2/E6E7 ratio analysis in the detection of HPV integration status, and may be used to select candidates for cervical biopsy and reduce the incidence of cervical cancer (43).

The present study identified 13 HPV52-positive patients by the analysis of 468 exfoliated cervical cell samples. Among them, 1 was purely episomal, 3 were purely integrated, and 9 were mixed (episomal and integrated). The results of 3 integrated HPV52 samples indicated that the integration of high-risk HPV may have an important role in cervical cancer. Furthermore, viral integration may occur in the E6 and E7 genes or downstream in the E1 or E2 area, causing gene inactivation (18,44,45). Integration of the viral genome is typically caused by an HPV E2 open reading frame that is broken or missing; in such cases, certain areas of the DNA fragment are lost, and the E7 or E6 gene is integrated into the host cell, which promotes and maintains the integration of the viral genome and the interaction with host genes (46).

Cytological analysis of the 13 HPV18-positive specimens according to the TBS indicated that there was 1 case of ASC, 5 cases of LSIL and 7 cases of HSIL. As the lesion stage advanced from ASC to LSIL and HSIL, the proportion of episomal samples decreased, and that of integrated samples

### Table II. Distribution of viral load and integration state of HPV52.

| Cytodiagnosis | Viral load (copy no./cell) | No. of cases | HPV52 integration state, n (%) |
|---------------|----------------------------|-------------|-------------------------------|
|               |                            |             | Episomal | Mixed | Integrated |
| ASC           | 23.26                      | 1           | 1 (100.0) | 0     | 0          |
| LSIL          | 9.88-189.25                | 5           | 0       | 4 (80.0) | 1 (20.0) |
| HSIL          | 19.52-98.52                | 7           | 0       | 5 (71.4) | 2 (28.6) |
| Total         | 9.88-189.25                | 13          | 1 (7.7) | 9 (69.2) | 3 (23.1) |

*Mixture of episomal and integrated virus. HPV, human papilloma virus; ASC, atypical squamous epithelial cells; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.*

Figure 3. Cytological diagnosis of cervical lesions. (A) Atypical squamous cells, (B) low-grade squamous intraepithelial lesions and (C) high-grade squamous epithelial lesions. (A) The arrowhead indicates the cell volume increased, the cytoplasm was rich, the nucleus was slightly enlarged and nuclear polarity was lacking in the atypical squamous cells. (B) The arrowhead indicates koilocyte was visible near the edge and in the middle of the field of view, with an atypical concave nuclear space, an irregular nuclear membrane, a visible dual-core, a free halo around the nucleus and a stiff surrounding halo, with an iron wire mesh-like shape. (C) The arrowhead indicates the cell was alone with a larger nucleus, the cytoplasm exhibited keratosis and the cytoplasmic area was decreased. Magnification, x400.
increased for the HPV52-infected patients. The results indicate that pathological changes are associated with integration status, and the 3 HPV52-positive samples integrated into human chromosome 2, 5 or 8. This is consistent with previous results, which have suggested that integration of the viral genome increases the severity of the disease, at least as indicated by biomarker studies of HPV16-infected cervical lesions and precancerous lesions (38,32,47). Therefore, a persistent
infection following integration of the viral genome into human host cell chromosomes may be key for cell transformation and cancer development.

HPV52 integration into the host genome may be a key factor in cervical lesions, therefore, patients at high risk for cervical lesions may potentially be identified by screening for HPV52 infection and integration.

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