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Genomic Organization of Human Papillomavirus Type 18 in Cervical Cancer Specimens

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It has been reported that cervical cancers positive for human papillomavirus (HPV) 18 have a poorer prognosis than those with other HPV types. To gain a better understanding of the aggressive character of HPV 18-positive cancers, we examined the difference in genomic organization between HPV 18 and HPV 16 harbored in cervical cancers. We amplified E1 and E2 genes from 9 HPV 18-positive and 31 HPV 16-positive cervical cancers by polymerase chain reaction (PCR). At least one of the two early genes was missing in 3 out of 9 HPV 18-positive cancers, while both PCRs were positive in all 31 HPV 16-positive cancers ($P<0.05$). We then analyzed the 9 HPV 18-positive cancers by 15 contiguous polymerase chain reactions covering collectively the whole HPV 18 genome. In addition to the three with a deletion of the E1 or E2 gene, one had a deletion in the E5 and L2 genes and one had an insertion in the long control region. The frequent alterations in genomic organization, especially preferential deletion of the E1 or E2 gene, may be related to the more aggressive properties of HPV 18-positive cervical cancers.

Key words: Human papillomavirus type 18 — Cervical cancer — Polymerase chain reaction

Cervical cancer is one of the most common malignant diseases in women worldwide. Certain types of human papillomavirus (HPV) such as types 16, 18, 31, 33, 35, 45, 52, 56 and 58 appear to play an important role in the development of cervical cancer. Among cervical cancer-associated HPV types, HPV 16 and 18 are frequently identified in cervical cancer specimens and have been studied intensively.

HPV 18 is preferentially associated with aggressive cancers of the cervix and cervical cancer patients with HPV 18 have a poorer prognosis than those with other HPV types.[1-3] A more aggressive transforming activity of HPV 18 has not been seen, compared with that of HPV 16, in *in vitro* studies.[4-6] Therefore, one may find a clue to the aggressive nature of HPV 18 by examining how the viral genome resides in the cancer cells. Both episomal and integrated forms of the HPV genome are usually present in cancer cells containing HPV types 16, 31, 33, 35, 45, 52, 56 and 58.[7-11] However, the presence of a completely or an incompletely integrated genome without episomal forms has been shown in HPV 18-positive cancer specimens.[8, 10, 12, 13] It is also interesting to note that only integrated forms are present in permanent cell lines established from either HPV 16- or 18-positive cancers.[13-17] Since the establishment of cell lines is indicative of higher tumorigenicity, it is possible that the loss of the episomal genomes leads to a more aggressive phenotype. HPV genomes integrated in the chromosome DNA are frequently disrupted at the E1 or E2 gene in established cell lines.[14, 17, 18] Disruption and functional loss of the E2 gene are consistent with a more aggressive phenotype, since the E2 gene product behaves as a repressor for the E6 and E7 oncogenes.[19-22] So far, the site of disruption or deletion in the HPV 18 genome from cancer specimens has not been extensively studied. We used the polymerase chain reaction (PCR) to examine whether the E1 or E2 gene is deleted in HPV 18- and 16-positive cancer specimens. Subsequently, to examine deletion or disruption of other genes, we chose PCR primers such that the whole HPV 18 DNA could be amplified as 15 contiguous fragments (whole-genome PCR).

MATERIALS AND METHODS

Materials The biopsy specimens studied here included 12 HPV 18-positive cervical neoplasias (3 intraepithelial neoplasias grade 3 [CIN III] and 9 cancers) and 40 HPV 16-positive cervical neoplasias (9 CIN III and 31 cancers). The mean age (range) of these groups was as follows; 37.0 (27–47) yr in HPV 18-positive CIN III, 41.6 (24–75) yr in HPV 18-positive cancer, 36.9 (27–49) yr in HPV 16-positive CIN III and 57.1 (26–74) yr in HPV 16-positive cancer. The patients with HPV 18-positive cancer had a poorer prognosis than those with

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HPV 16-positive cancer (44.4% vs. 80.6% 3-year survival), although both cancers had almost the same distribution of clinical stages. Detection and typing of HPV DNA in these specimens were carried out by means of consensus sequence-primed PCRs of the E6 gene and the L1 gene (E6-PCR and L1-PCR) and subsequent restriction enzyme digestion as described previously.[23,24] Typing of HPV 18 and HPV 16 was consistent in both consensus sequence-primed PCRs.

HPV types 16 and 18 cloned in pBR 322 were linearized with restriction enzymes and used to determine the specificity and sensitivity of the PCR systems.

To evaluate our whole-genome PCR, we used HeLa cells whose HPV 18 genome structure is known and a condyloma containing episomal forms with complete HPV 18 DNA (data not shown).

PCR primers for the E1 gene and the E2 gene of HPV 18 and HPV 16 Sets of primers targeting a portion of the E1 gene and E2 gene of HPV 18 and 16 DNA were used (18E1-, 18E2-, 16E1- and 16E2-PCRs). The PCRs have the same size of product (250 bp) and a slightly higher level of sensitivity than E6-PCR and L1-PCR (data not shown). The nucleotide sequences (base position in the GENBANK and EMBL data bases) of the sense and antisense primers for each PCR are as follows; 18E1-PCR, 5′-GCAGACAGCAACAGCAATGC-3′ (2031–2050) and 5′-TGGTATCGCAGGAATTGCAC-3′ (2280–2261), 18E2-PCR, 5′-GGCCAAACAGTACAAGTATA-3′ (3168–3187) and 5′-TACAATCAATTACATTATTC-3′ (3417–3398), 16E1-PCR, 5′-ACAATGGGCCTACGATAATG-3′ (1946–1965) and 5′-CATAACAATTTGCTTCCAAT-3′ (2195–2176) and 16E2-PCR, 5′-TGCGCCTAGAATGTGCTATT-3′ (2861–2880) and 5′-ACTTCCACTGTATATCCATG-3′ (3110–3091). These primers were synthesized by a model 380B DNA synthesizer (Applied Biosystems, Tokyo).

Whole-genome PCR primers for HPV 18 Oligonucleotide primers were chosen so that the whole HPV 18 genome could be amplified as 15 fragments (A to O) (Fig. 2). We designed the system so that each fragment overlapped with both neighboring fragments in the sequences of the primers. The size of each fragment was 502 to 608 bp. The PCRs have a slightly higher level of sensitivity than E6-PCR and L1-PCR (data not shown). The base positions of sense and antisense primers for each PCR are shown, according to the sequences in the GENBANK and EMBL data bases, in Fig. 2. These primers were synthesized by use of a model 380B DNA synthesizer (Applied Biosystems).

PCR The PCR protocol employed in this study was 40 cycles of denaturation (95°C, 1.5 min), annealing (55°C, 1.5 min) and extension (70°C, 2 min) and was performed on a BioGene PHC-1 (Techne Ltd., Cambridge, England). Each reaction mixture of 100 μl contained 50 m*M* KCl, 10 m*M* Tris-Cl pH 8.4, 1.5 m*M* $MgCl_2$, 200 μM of each NTPs, 1 μM of each primer pair and 4 units of Taq polymerase (Biotech International Ltd., Bentley, WA). PCR reaction was carried out with 0.1 μg of cellular DNA extracted from biopsy specimens according to the standard procedure. A 10 μl aliquot of the reaction mixture was subsequently electrophoresed on 4% NuSieve GTG agarose gel containing 1 μg/ml ethidium bromide. PCR was judged positive if a single band of the expected size was detected.

The sensitivity of the 18E1-, 18E2-, 16E1- and 16E2-PCRs and the whole-genome PCR (fifteen PCRs) was assessed by serial dilution of cloned HPV 18 and HPV 16 DNA (10^{-2} pg, 1 fg and 10^{-1} fg) mixed with 0.1 μg of placental DNA. In this assay, all PCRs except PCR for fragment O, which contains an *Eco*R I site for cloning, were able to detect 1 fg of cloned HPV DNA in 0.1 μg of placental DNA. In the assay using serial dilution of a condyloma DNA sample containing episomal HPV 18 DNA, the fragment O was amplified as efficiently as the other fragments.

RESULTS

Comparison of HPV 18- and HPV 16-positive cervical neoplasias by PCRs for the E1 gene and the E2 gene All 3 HPV 18-containing CIN III were positive in 18E1- and 18E2-PCRs and all 9 HPV 16-containing CIN III were positive in 16E1- and 16E2-PCRs (Table I).

While 6 of 9 HPV 18-containing cancers were positive in 18E1- and 18E2-PCRs, 3 cancers were negative in 18E1-PCR, 18E2-PCR or both, respectively (cases 10, 11 and 12 in Fig. 2). In contrast, all 31 HPV 16-containing cancers were positive in both 16E1- and 16E2-PCRs (Table I). The difference in amplification of the E1 and E2 genes between HPV 18- and HPV 16-positive cancers was significant (the chi-square statistic, $P<0.05$).

Analysis of HPV 18 DNA by whole-genome PCR in CIN and cancers We tested cell DNAs from a condyloma containing episomal HPV 18 DNA and HeLa cells whose integrated HPV 18 genome structure is known. All 15 fragments were amplified in DNA from the condyloma and fragments G to O were amplified, but not fragments A to F in HeLa cell DNA (Fig. 1, Fig. 2). This result was consistent with the published HPV 18 genome structure in HeLa cells, with a large deletion spanning the E2, E5, L2 and L1 regions,[18] and confirms that whole-genome PCR can be used to identify disorganization in the viral genome.

All 15 fragments were amplified in three HPV 18-positive CIN III (cases 2 to 4 in Fig. 2), as well as in the above condyloma.

All 15 fragments were amplified in 5 of 9 samples (case 5 to case 9 in Fig. 2). In one of these 5 samples (case 9),

Table I. Negative Reactions on PCR[a] for the E1 and E2 Genes by HPV Type and Histological Diagnosis

Histology	Type 18	Type 16
CIN III	0/3	0/9
Cancer	3/9[b]	0/31[b]

a) 18E1-, 18E2-, 16E1- and 16E2-PCRs were used.
b) χ^2 for HPV 18-positive cancer versus HPV 16-positive cancer; $P<0.05$.

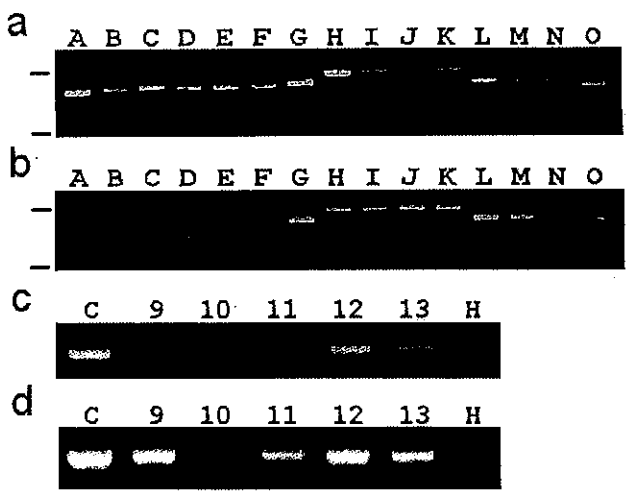

Fig. 1. Amplification of HPV 18 DNA sequences using whole-genome PCR in control samples (a, b) and cervical cancer samples (c, d). In a and b, bars on the left side show molecular markers ($\phi\times174$ digested with *Hae* III): from the top, 603 and 310 base pairs. a, Positive reactions in PCRs for fragments A to O in a condyloma sample containing episomal HPV 18 DNA. b, Negative reactions in PCRs for fragments A to F and positive reactions in PCRs for fragments G to O in HeLa cells. In c and d, the lane numbers correspond to the case numbers in Table II and Fig. 2. Lane C was the above condyloma sample as a positive control. Lane H was HeLa cells as a negative control. c, fragment A (508 bp) was not amplified in lanes 10, 11 and H. d, fragment B (517 bp) was not amplified in lanes 10 and H.

the product amplified by PCR for fragment J was larger. On restriction cleavage of the fragment, an insertion of a 430 bp sequence was found at the 3′ end of the long control region (LCR). A region covered by contiguous fragments was not amplified in 4 of 9 samples. Three samples had a deletion containing E1 or E2 gene and one had a deletion of other genes (the E5 and L2 genes) (Fig. 1, case 10 to case 13 in Fig. 2). Therefore, early regions around the E1 and E2 genes appeared to be most frequently deleted in HPV 18-positive cervical cancer cells, while L1, E6 and E7 genes and LCR were retained.

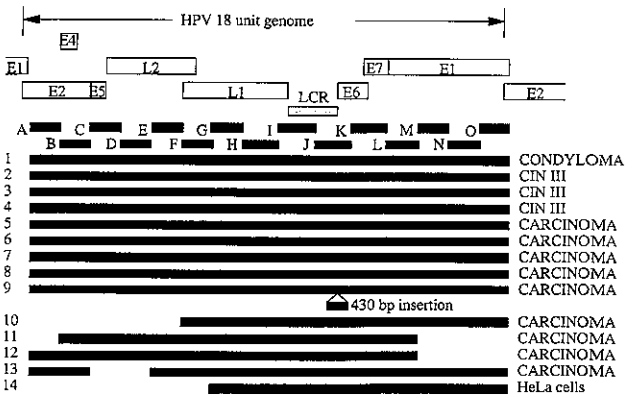

Fig. 2. The sequences amplified by whole-genome PCR in HPV 18-positive cervical neoplasias (1, condyloma; 2–4, CIN III; 5–13, invasive carcinomas; 14, HeLa cells). Open boxes indicate open reading frames (ORFs) in the HPV18 unit genome. The stippled box indicates the long control region (LCR). Short solid boxes show the locations of the target fragments (A to O) of the whole-genome PCR reaction. Long solid boxes indicate the amplified sequences. Base positions of sense and antisense primers for each PCR are as follows: A (2910–2929, 3417–3398), B (3398–3417, 3914–3895), C (3895–3914, 4417–4398), D (4398–4417, 4919–4900), E (4900–4919, 5421–5402), F (5402–5421, 5903–5884), G (5884–5903, 6425–6406), H (6406–6425, 7012–6993), I (6993–7012, 7597–7578), J (7578–7597, 325–306), K (306–325, 913–894), L (894–913, 1423–1404), M (1404–1423, 1932–1913), N (1913–1932, 2441–2422), O (2422–2441, 2929–2910).

The gene alteration of HPV DNA and the outcome after primary treatment with surgery or radiotherapy in 9 individual cases with HPV 18-positive cancer are shown in Table II. Three of 5 (60%) cases with gene alteration (cases 9 to 13) died of cancer within 3 years after primary treatment, while 2 of 4 (50%) cases without gene alteration (cases 5 to 8) died of cancer during the same period. Of the latter cases, one who died (case 5) had an atypical *Pst* I-digestion pattern and one who remained alive (case 6) had a typical *Pst* I-digestion pattern in our previous Southern blot analysis,[8] although all 15 fragments were amplified by the whole-genome PCR in both cases. The dominant HPV 18 DNA in case 5 did not appear to be the full-length HPV genome.

DISCUSSION

PCRs for the E1 or E2 gene (18E1-, 18E2-, 16E1- and 16E2-PCRs) were positive in all HPV 18- and HPV 16-positive cervical precancerous lesions (CIN). HPV DNA is usually maintained as an episome in benign productive infections and CIN.[10,11] Our data are compatible with those reports.

Table II. Gene Alteration of HPV 18 DNA and Outcome of Cases with HPV 18-Positive Cervical Cancer

Case No.	Age	Stage	Histology	Southern blot analysis (*Pst* I-digestion pattern)	Deleted genes	Outcome	
5	36	1b	A	atypical	None	DOD	33 mo
6	34	2b	A	typical	None	NED	71 mo
7	24	2b	S	(—)	None	DOD	10 mo
8	75	3b	S	NT	None	NED	39 mo
9	66	1b	S	NT	LCR (insertion)	DOD	6 mo
10	41	1b	S	NT	E2, E4, E5, L2	NED	36 mo
11	37	1b	S	(—)	E1, E2	DOD	37 mo
12	36	2b	AS	NT	E1	NED	49 mo
13	25	2b	S	atypical	E5, L2	DOD	13 mo

Abbreviations: A, adenocarcinoma; S, squamous cell carcinoma; AS, adenosquamous carcinoma; (—), HPV DNA was not detected; NT, not tested; LCR, long control region; DOD, died of disease; NED, no evidence of disease.

As to cervical cancers, 16E1- and 16E2-PCRs were positive for all 31 HPV 16-positive cancers, while 18E1- and 18E2-PCRs were positive only for 6 of 9 HPV 18-positive carcinomas ($P<0.05$). Recently, Berumen *et al.*[12] reported that the E1/E2 region was absent in all tumors positive for HPV 18 and present in 64% of those positive for HPV 16, also using a PCR assay. Their PCR for E1/E2 might have lower sensitivity than that for E6/E7, because their primers flanked a 0.8-kb fragment for E6/E7 and a 2.9-kb fragment for E1/E2. In this sense, our results on E1/E2 status are consistent with their report.

It has been reported that almost all HPV 16-positive cancers have a full viral genome in an episomal form or a multimeric integrated form on Southern blot analysis using multi-cut enzymes.[9, 10] We also reported that all 19 HPV 16-positive cancers showed typical *Pst* I-digestion patterns and 11 of the 19 had episomal HPV 16 DNA.[8] On the other hand, it was reported that in 11 of 14 HPV 16-containing cancers, deletions and/or rearrangements were detected in the E1 and E2 genes by Southern blot analysis with probes for HPV 16E1, E2, E6, and E7 genes.[25] The reason for this discrepancy may be that the criteria for the assignment of HPV types was stricter in the former three papers than in the latter paper. The possibility that the HPV 16-positive samples in the latter paper included cancers containing highly homologous HPV types to HPV 16 such as HPV 31, 33, 35, 52 and 58 cannot be excluded, since the criteria for assignment of HPV types were strength of hybridization and restriction patterns compared only with those of HPV types 6, 11, 18 and 31. Our data are consistent with the former three papers, suggesting that almost all HPV 16-containing cancers have a full viral genome.

In contrast, HPV 18-containing cancers tend to show diverse cleavage patterns on Southern blot analysis even in highly stringent conditions, and the consistent integration of HPV DNA into the host genome has been revealed.[10, 13] We reported previously that 2 of 3 HPV 18-positive cervical cancers showed atypical *Pst* I-digestion patterns, and only one showed a typical pattern.[8] These 3 HPV 18-positive samples were also enrolled in this study (cases 5, 6 and 13 in Table II). However, the site of disruption or deletion has so far been unclear. The present data suggest that HPV 18-positive cancers frequently have viral genomes disrupted or deleted in the E1 or E2 gene.

Subsequently we used whole-genome PCR for HPV 18 DNA (Fig. 1). The whole-genome PCR was evaluated by an analysis of cell DNAs from HeLa cells, whose HPV 18 genome structure is known, and from a condyloma containing episomal forms of complete HPV 18 DNA. All 15 fragments were amplified in all three HPV 18-containing CIN III. These data suggest that HPV 18-positive precancerous lesions contain the full-length HPV genome.

Of 9 HPV 18-positive cancers, 3 were lacking a region covering two or more fragments containing the E1 or E2 gene, in accordance with the results of 18E1- and 18E2-PCRs, and another was devoid of a region covering E5 and L2. Though it is possible that the lack of amplification on two contiguous fragments is brought about by an interruption and integration event within a primer sequence, the lack of amplification of three or more contiguous fragments strongly suggests a deletion in the HPV genome. Hecht *et al.*[26] reported that the HPV 18 E2 gene from 2 of 5 cancer tissue specimens was not amplified by PCR. The gene alterations, especially deletion of E1 or E2 gene, frequently observed in HPV 18 DNA may promote rapid progression to aggressive malignancy, though there remains a possibility that the

aggressive properties are caused by HPV 18 gene function itself and the gene alterations are not the cause, but just the result.

The association between the gene alteration of HPV DNA and survival was not so apparent in the small number of cases with HPV 18-positive cancer (Table II). However, the gene alterations of HPV DNA that are observed frequently in HPV 18-positive cervical cancers might explain the poorer prognosis, because these gene alterations rarely occur in cervical cancers containing other HPV types. It is worth studying further the relationship of the gene alteration of HPV DNA and the prognosis in individual cases with cervical cancer.

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