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Structural and Expressional Alterations of Episomal and Integrated Human Papillomavirus Type 16 in Precancerous Lesions and Carcinomas of the Cervix.

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ABSTRACT

HPV infection has long been implicated in the development of cervical carcinoma. We have analyzed the HPV 16 genome structure and expression of the viral mRNA in cervical intraepithelial neoplasias (CINs) and cervical carcinomas by using modified polymerase chain reaction (PCR) methods. Genome structure has been determined by PCR using multi-primer sets which are located in each open reading frames and then these results have been compared with the physical state of the viral DNA determined by two-dimensional electrophoresis. Furthermore, we have analyzed the expression of HPV 16 mRNA and genome structure using DNA and RNA simultaneously extracted from CINs and cervical carcinomas using PCR and reverse transcription (RT-) PCR.

Our data showed that the DNA regions from the E1 to L1 region were deleted in two of three CINs containing episomal HPV 16 and three out of seven cervical carcinomas containing integrated HPV 16. However, the E6/E7 region was conserved in all the HPV 16-positive samples.

RT-PCR analysis has determined the presence of mRNA species which could encode the E6, E6*I, E6*II, E7, E2, E2•C, E1^E4, E1^E2•C, E4, E2•C-E5 and L2 proteins. The overall results of DNA and mRNA analyses in cervical lesions indicated that the expression patterns of the early and late transcripts studied were not specifically related to the grade of malignancy and the physical state or the deletion of the viral genome. Furthermore, alterations in the splicing pat-

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terns of HPV 16 transcripts may not be involved in tumor progression.

Key words: Human papillomavirus 16 (HPV 16), Polymerase chain reaction, Physical state, Genome deletion, mRNA, Reverse transcription,

INTRODUCTION

There is a strong association between certain types of human papillomavirus (HPV) and cervical carcinoma(1). In particular, HPV type 16 (HPV 16) is the most frequently found among the oncogenic HPVs, which are present in approximately 80% of cervical carcinomas and 70% of cervical intraepithelial neoplasias (CINs)(2). The E6-E7 region of HPV 16, which is invariably present in cervical carcinomas and CINs, encodes proteins that are capable of transcriptional regulation and cellular transformation through interaction with cellular proteins, such as the tumor suppresser gene products pRB and p53(1).

HPV 16 is often found integrated into the tumor cell genome, although it may also persist as extrachromosomal episomes or mixtures of episomal and integrated DNAs(2, 3). The presence of integrated HPV DNA frequently found in CINs, cervical cancers and immortalized keratinocytes has suggested that viral integration may be an important step in the viral transformation of infected cells (2, 3). In contrast, precancerous and benign cervical lesions predominantly contain episomal HPV DNA.

The E6-E7 region of HPV 16 is consistently conserved upon viral genomic integration into the cellular genome. Since E1 and E2 genes are responsible for the correct episomal replication and regulation of the expression of the viral genes, integration of the viral genome, accompanied by deletion of E1 and E2 regions, may lead to unregulated expression of the transforming genes E6 and E7 (4).

Analysis of HPV 16 mRNA in CIN tissues is often difficult because only a small specimen amount is available. The most sensitive procedure for the detection of mRNA has been provided by the combination of reverse transcription and the polymerase chain reaction (RT-PCR). By using the RT-PCR method, several reports have analyzed the splicing patterns of E6 and E7 mRNAs in cervical lesions(5-8).

In this study, we first analyzed the physical state of HPV 16 in cervical carcinomas and CINs, and compared the results of Southern blot hybridization following two-dimensional gel electrophoresis(2) with PCR by using multiple primer sets which targeted open reading frames (ORFs) for amplification. Second, we analyzed the expression of the HPV 16 genome by using the RT-PCR method. Furthermore, we compared the expression and the genome structure of HPV 16

using the RT-PCR and PCR methods.

MATERIALS AND METHODS

Materials

Tissue specimens were taken from cervical lesions while patients were in the operating room and the outpatient department of Sapporo Medical University Hospital. Twenty-nine specimens were frozen immediately by liquid nitrogen and stored at −70°C. A part of the tissue was fixed in 10% formalin and diagnosed histopathologically. The presence of HPV 16 DNA in these cases was confirmed by Southern blot hybridization(2), PCR methods(9-11) and direct sequencing method(12) as previously described.

Extraction of DNA

Twenty frozen biopsy samples were minced and suspended in 0.1 M EDTA, 0.2 M Tris HCl and 1% SDS. These samples were digested with proteinase K at 37°C for 12-16 hr; the total cellular DNA was extracted with phenol/chloroform and precipitated with two volumes of ethanol. The DNAs were further treated with 50 μg/ml of RNAse A at 37°C for 1 hr and extracted with phenol/chloroform. After precipitation with ethanol, samples were dissolved in 10 mM of Tris HCl and 1 nM of EDTA (pH 8.0) and stored at 4°C.

Simultaneous extraction of DNA and RNA

Nine tissue specimens were minced and homogenized quickly in 4M guanidium isothiocyanate−25 mM sodium citrate (pH 6.0)−1% 2-mercaptoethanol (GIT buffer)(13). The homogenates were layered onto a cushion of 5.7 M CsCl-0.1 M EDTA (pH 7.5) in Beckman SW41 tubes and centrifuged at 33,000 rpm for 18 hr at 20°C. During centrifugation, the RNA formed a pellet on the bottom of the tube while most of the DNA and protein floated upward in the CsCl solution. The DNA was recovered from the solution and dialyzed against distilled water. Then the DNA was purified by phenol/chloroform extraction and ethanol precipitation. The RNA pellet was purified by ethanol precipitation.

Two-dimensional gel electrophoresis and hybridization

We used two-dimensional gel electrophoresis to clarify the physical state of the HPV 16 DNA as described before(2). In 10 cases of cervical carcinoma and 4 cases of CIN, linear and circular DNA could be distinguished.

Synthesis of first-strand cDNA

Reverse transcription was carried out in a 30 μl reaction mixture consisting

of 50 mM Tris-HCl (pH 8.3), 100 mM KCl, 10m M $MgCl_2$, 10 mM DTT, 1 mM each dNTP, 65 units of RNase inhibitor (Takara), 10 units of reverse transcriptase (RAV-2, Takara), 5 μg of oligo dT and approximately 1 μg of denatured total RNA. The reaction mixture was incubated for 1 hr at 42°C, then the reverse transcriptase was inactivated at 95°C for 5 min.

To confirm the absence of DNA in RNA samples, total RNA was subjected to PCR with primers p16-3 and 6R, which can amplify the HPV 16 sequence only from genomic DNA.

PCR primers for detection of HPV 16 DNA and mRNA

For analysis of the structure of the HPV 16 genome and the species of HPV 16 mRNA in cervical lesions, multiple primer sets to amplify each ORF of HPV 16 were synthesized according to the sequence determined by Seedorf *et al.* (14) (Table 1).

PCR

Amplification of target HPV cDNA or approximately 1 μg of DNA from tissue specimens was performed using the PCR method in a 100 μl mixture containing 1/15 volume of 1st strand cDNA (2 μl) or DNA, 10 μl of 10 X PCR buffer (100 mM Tris-KCl (pH 8.3), 500 mM KCl, 1.5 mM $MgCl_2$, 0.1% (w/v) gelatin), 16 μl of dNTP solution (1.25 mM in each dNTP), 20 pmol of each HPV 16 specific primer pair (Table 1), and 2.5 units of Taq DNA polymerase (Perkin Elmer). The mixture was overlaid with 75 μl of mineral oil and subjected to 25 or 30 cycles of PCR in a DNA Thermal Cycler (Perkin Elmer). Each PCR cycle

Table 1 *Position of the oligonucleotides primers used in PCR and RT-PCR. The nucleotide numbers correspond with HPV 16 sequences reported by Seedorf et al.* (14)

HPV16 ORF	Primer		Nucleotide localization	Length (bp)
	sense	anti-sense		
LCR-E6	p16-1	p16-2R	26-165	140
E7-E1	p16-5	p16-9R	661-1003	343
E1	p16-10	p16-12R	1270-1826	557
E1-E2/E4	p16-14	p16-15R	2753-3375	623
E2	p16-16	p16-17R	3386-3711	326
E2・C-E5	p16-16	p16-17R	3386-3891	506
E2-L2	p16-18	p16-20R	3765-4247	483
L2	pL2-2	pL2-3R	4403-4665	263
L1	pL1-1	pL1-2R	5961-6202	242

included 1 min. of denaturation at 94°C, 2 min. of primer annealing at 55°C, and 2 min. of primer extension at 72°C. Then 10 μl of each reaction mixture was analyzed on an ethidium bromide-stained 3 % NuSieve and 1% SeaKem agarose (FMC) gel.

Cloning and sequencing

Amplified cDNA was purified by phenol/chloroform extraction, ethanol precipitation and blunt ended with a DNA blunting kit (Takara). We electrophoresed the PCR products in 3% NuSieve GTG agarose (FMC) gel, cut out the desired segment of gel and heated it at 65°C to melt the gel. Then the agarose was removed by phenol/chloroform extraction and purified by ethanol precipitation. Amplified cDNA fragments were ligated to the HincII site of the M13mp18 phage with T4 DNA ligase (Takara) for 16 hr at 15°C. The plaques of M13 which contained the cDNA fragment were selected by the method reported by Messing(15) and 1st-strand cDNA was prepared. Sequencing was performed using the 1st-strand cDNA by the dideoxy method(16).

RESULTS

Typing of HPV

To confirm the type of HPV in the samples for this study, we utilized Southern blot hybridization(2), type specific primers(9) and universal primers for PCR (10, 11). Furthermore, direct sequence analysis of the E7 region was done(12). These data made it clear that the type of HPV in our samples in this study was HPV 16.

Physical state of HPV 16 in cervical carcinomas and CINs

Ten specimens of cervical carcinoma with HPV 16 DNA were examined to determine their physical state using two-dimensional gel electrophoresis(2). Episomal HPV DNA could be seen as discrete hybridization signals off the track of linear DNA as these molecules had decreased electrophoretic mobility in the second dimension. Nine of the ten carcinomas showed an integrated or integrated plus episomal pattern, whereas the remaining one showed only an episomal state(2) as summarized in Table 2. Two-dimensional gel electrophoresis was also performed for the analysis of the physical state of HPV 16 DNA in CIN. Three of four CINs were shown to have episomal HPV 16 DNAs and one was shown to have an integrated genome(2) as shown in Table 2.

Amplification of HPV 16 genome

To analyze the structure of the HPV 16 genome, we used 6 or 7 sets of

Table 2 *Summary of the data about the physical state determined by two dimensional electrophoresis and the deletion of HPV 16 genome analyzed by PCR : The PCR results are compared with physical states which determined by two dimensional electrophoresis* (2). *I* : Integrated, E* : Episomal, All Positive*** : All of the regions were amplified by PCR, Some Negative**** : Some of the regions were not amplified by PCR, N. D.**** : Not determined*

Sample	Physical State	All Positive***	Some Negative****	Total Cases
Carcinoma	Both I* and E**	2	0	2
	I	4	3	7
	E	1	0	1
	N. D.*****	3	1	4
CIN	I	1	0	1
	E	1	2	3
	N. D.	2	0	2

specific PCR primers for each ORF of HPV 16 (Fig. 1). Fourteen cervical carcinomas and 6 CINs, the physical states of which were determined by two-dimensional electrophoresis (2), were subsequently examined by PCR. Figure. 2 shows the gel electrophoresis of the PCR products from 2 carcinomas and 2 CINs as representatives for all samples. Ca80 contained both episomal and integrated viral DNAs, and Ca89 contained only the integrated viral DNA. Figure. 2A

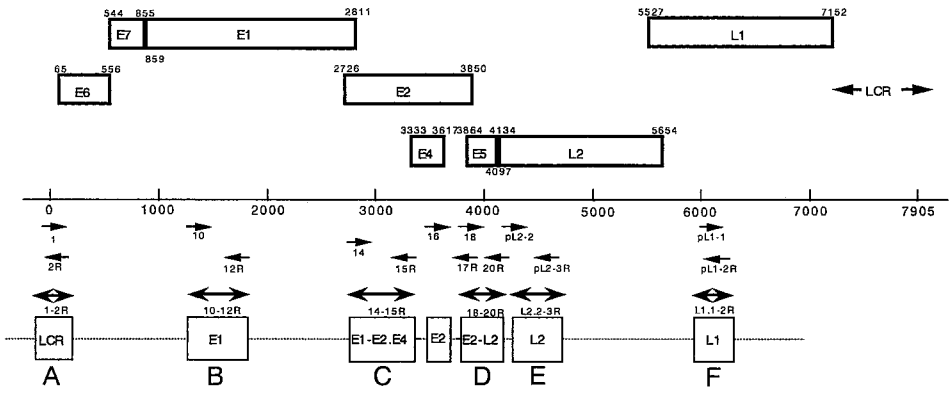

Fig. 1 Locations of primers for analysis of HPV 16 genome. Upper lane shows the genomic map of HPV 16 which was determined from analysis of the DNA sequence by Seedorf *et al.* (14). Nucleotide numbers are noted on the each ORFs. Lower lane shows the location of primers and amplified products.

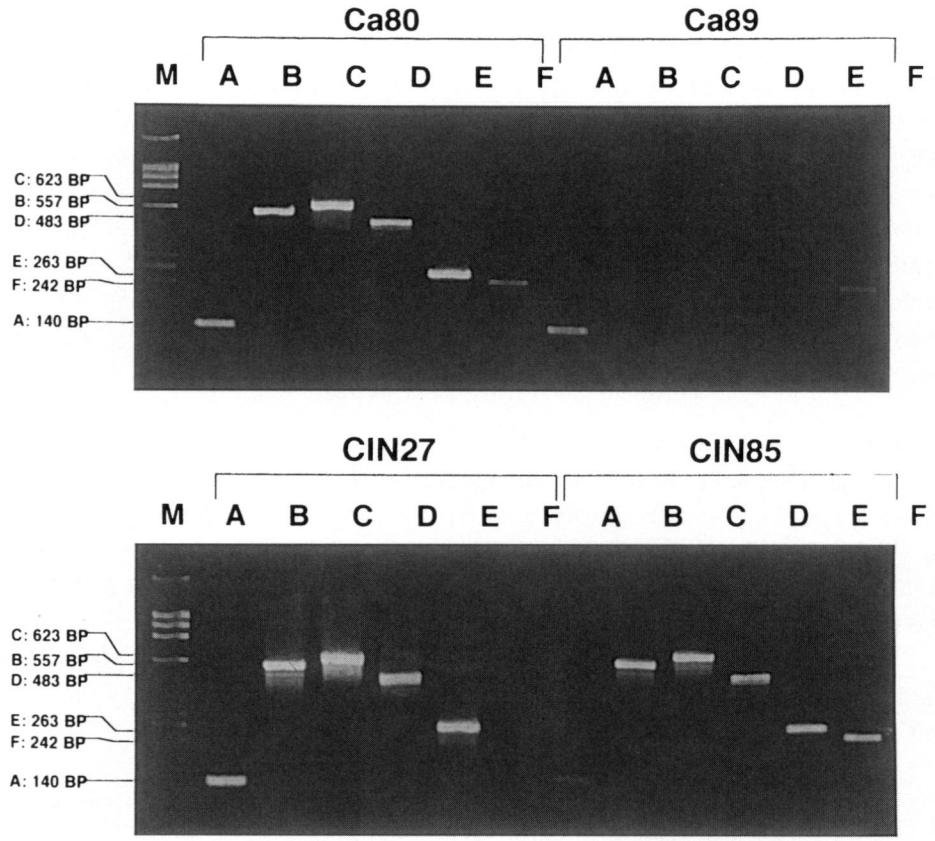

Fig. 2 Gel electrophoresis of the PCR products from 2 carcinomas and 2 CINs. M: Marker Lane A-F represent the amplified products shown in Fig. 1.(A) Cervical carcinoma samples. Although all the regions from LCR to L1 are amplified in Ca 80 (lane A-lane F), the regions from E1 to L2 (lane B-lane E) are not amplified in Ca89. (B) CIN samples. L1 region (lane F) is not amplified in CIN27, however all the regions are amplified in CIN85.

revealed that all the regions from LCR to L1 were amplified from Ca80 (lane A-lane F), while the regions from E1 to L2 (lane B-lane E) were not amplified from Ca89. As shown in Fig. 2B, the L1 region (lane F) was not amplified from CIN27, while all the regions were amplified from CIN85. Contrary to a previous report on the physical state, in which both samples contained episomal HPV DNA(2), the L1 region was considered to be deleted in CIN27.

In 10 of 14 cervical carcinomas and 4 of 6 CINs, all regions were amplified by PCR. However, some of the regions were not amplified in 4 cervical carcinomas and 2 CINs (Table 2). It is possible that deletion of the HPV 16

genome occurred in the regions which could not be amplified by PCR. In 4 of 7 cervical carcinomas and one CIN which contained only the integrated HPV 16 DNA, no disrupted or deleted region was detected by PCR. On the other hand, several regions were not amplified in 2 of 3 CINs which contained only episomal HPV 16 DNA (Table 2). This might suggest that genome deletion or mutation occurs in episomal HPV 16 DNA. Figure. 3 shows the deletion sites on the genome in each sample. In 3 of 7 cervical carcinomas which contained only integrated HPV 16 DNA, the E1-L2 or E1-E2 region was not amplified. It is suggested that the integration disrupted the region from the E1 to L2 ORF. Interestingly, viral DNA in 2 CINs persisted in an episomal state in spite of the fact that part of the genome was not amplified.

Expression of HPV 16 mRNA

Total RNA and DNA were simultaneously extracted from 3 cervical carcinomas and 6 CINs. The presence of HPV 16 in these samples was determined by PCR. Then RNA samples were analyzed by RT-PCR to examine the expression of viral mRNAs. Figure. 4 shows the locations of primers for RT-PCR and the map of mRNAs.

HPV 16 E6/E7 transcripts were detected in all HPV 16-positive cervical carcinomas and CINs. There was no significant difference in HPV 16 E6/E7 mRNA patterns between cervical carcinomas and CINs(5).

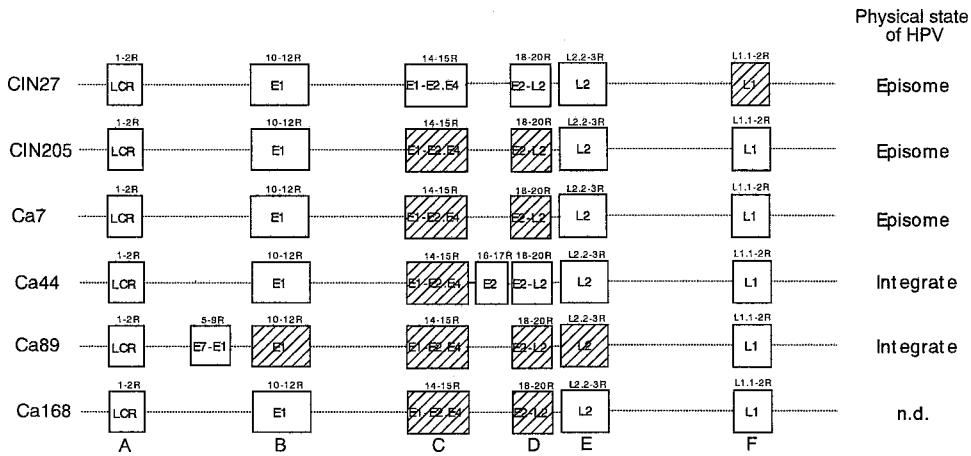

Fig. 3 Physical state and the location of HPV 16 genome deletion. Boxes represent the PCR amplified regions. Successful amplified regions are indicated by open boxes. Deleted regions which are not amplified by PCR are indicated by the slashed boxes.
n. d. : not determined

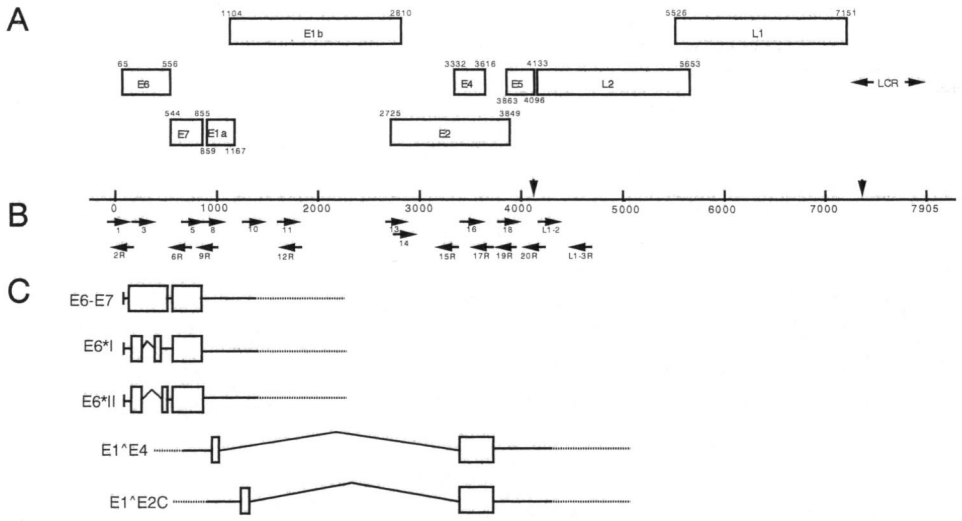

Fig. 4 The locations of HPV 16 specific primers and map of mRNA
A : Genomic organization of HPV 16. Polyadenylation signals are shown by arrow heads.
B : Locations of HPV 16 specific primers for RT-PCR
C : Map of HPV 16 mRNA

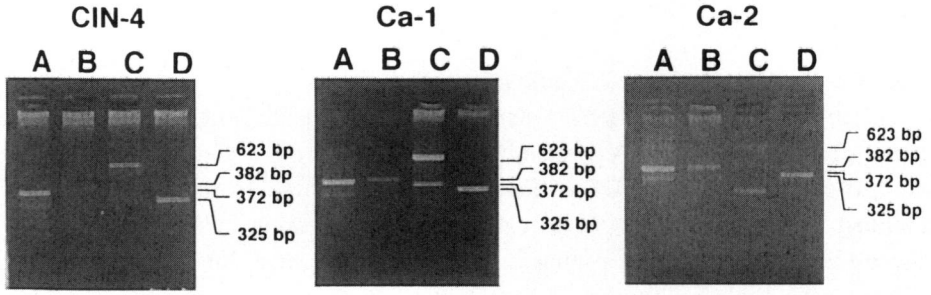

Fig. 5 Gel electrophoresis of the PCR products of E1^E4, E1^E2 • C, E2 • N and E2 • C
Ca-1, 2 : cervical carcinoma CIN-4 : CIN
Lane A : 372 bp of PCR product amplified by p16-8/17R (E1^E4)
Lane B : 384 bp of PCR product amplified by p16-10/17R (E1^E2 • C)
Lane C : 623 bp of PCR product amplified by p16-14/15R (E2 • N)
Lane D : 325 bp of PCR product amplified by p16-16/17R (E2 • C)

1) E1^E4, E1^E2 • C, E2 • N and E2 • C mRNA

To detect the spliced E1^E4 and E1^E2•C mRNAs, the 5′ region (E2•N) and 3′ region (E2 • C) of E2 mRNAs, we used p16-8/17R, p16-10/17R, p16-14/15R and p16-16/17R primer sets, respectively (Fig. 4). The sets primers, p16-8/17R

Table 3 *Expression of E1^E4, E1^E2・C, E2・N and E2・C mRNA in CINs and cervical carcinomas*

Case	E1^E4 p16-8/17R	E1^E2C p16-10/17R	E2・N p16-14/15R	E2・C p16-16/17R
CIN-4	++	+−	+	++
Ca-1	++	+	++	++
Ca-2	++	+	+	++

and p16-10/17R, are located upstream and downstream from splice donor sites of E1 mRNAs, respectively (Fig. 4, C). Figure. 5 shows the gel electrophoresis of each PCR product. In one CIN (CIN-4) and 2 cervical carcinomas (Ca-1, Ca-2), all the E1^E4, E1^E2・C, E2・N and E2・C mRNAs were expressed. These results are summarized in Table 3.

2) Splicing pattern of E1-E2 mRNA

Amplified PCR products using p16-8/17R and p16-10/17R were cloned and sequenced to determine the splicing sites. This showed that both transcripts were generated by splicing out introns in E1 and E2, from nucleotides (nt) 880 to 3357 in E1^E4 and from 1301 to 3357 in E1^E2・C as reported by Chow *et al.*(17). There was no significant difference in E1^E4 and E1^E2・C mRNAs between cervical carcinomas and CINs.

3) Expression levels of E1, E2 and E5 mRNA

To analyze the expression levels of E1, E2・C and E5 mRNA, we used p16-11 and p16-12R for E1 mRNA, p16-16 and p16-17R for E2・C mRNA and p16-18 and p16-19R for E5 mRNA. Furthermore, E7 mRNA was simultaneously amplified by p16-5 and p16-6R to estimate the expression level of each mRNA. Figure. 6 shows the gel electrophoresis of the PCR products of E1, E2・C and E5 mRNAs with the simultaneously amplified E7 product. There were 3 different patterns of these mRNAs (Table 4). In the first group (CIN-3 and Ca-2, 3), the 4 species of the mRNAs were equally expressed. In the second group (CIN-4 and Ca-1), E1 mRNA was less expressed than other mRNAs. In the third group (CIN-5, 6), E2・C and E5 mRNAs were not transcribed. There was no significant difference between cervical carcinomas and CINs in patterns of E1, E2・C and E5 mRNAs.

4) Expression of L2 mRNA

To analyze the expression pattern of L2 mRNA, we used pL2-2 and pL2-3R for L2 mRNA. The L2 mRNA was expressed in all six samples examined

(CIN-3, 4, 5, 6 and Ca-1, 2) (data not shown).

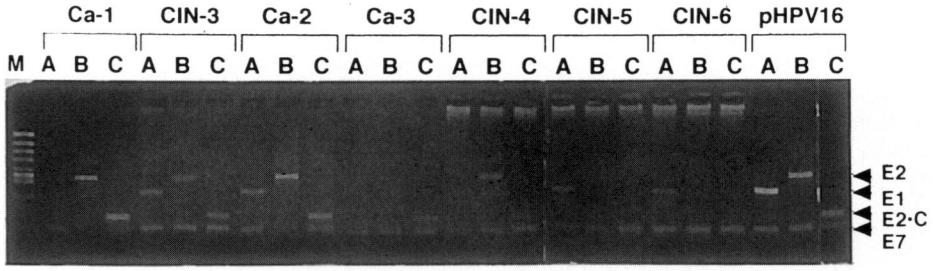

Fig. 6 Gel electrophoresis of amplified products of E1, E2・C and E5 mRNA with simultaneously amplified E7 product
M : Marker
Ca-1, Ca-2 and Ca-3 : cervical carcinoma sample
CIN-3, CIN-4, CIN-5 and CIN-6 : CIN sample
pHPV 16 : HPV 16 plasmid is used for positive control
Lane A : 232 bp of PCR product amplified by p16-11/12R (E1)
89 bp of PCR product amplified by p16-5/6R (E7)
Lane B : 325 bp of PCR product amplified by p16-16/17R (E2・C)
89 bp of PCR product amplified by p16-5/6R (E7)
Lane C : 127 bp of PCR product amplified by p16-18/19R (E5)
89 bp of PCR product amplified by p16-5/6R (E7)

Table 4 *Expression level of E1, E2・C and E5 mRNA in CINs and cervical carcinomas*

Case	E1 p16-11/12R	E2・C p16-16/17R	E2・C-E5 p16-18/19R	E7 p16-5/6R
CIN-3	+	+	+	+
CIN-4	+−	++	++	++
CIN-5	+	−	−	++
CIN-6	+	−	−	++
Ca-1	+	++	++	++
Ca-2	++	++	++	++
Ca-3	+−	+−	+−	+−

mRNA expression and genome deletion

To analyze the relationship between the genome deletion of HPV 16 and the expression of HPV 16 mRNA in CIN, we examined both DNAs and mRNAs that were simultaneously extracted from 2 CIN samples (Table 5). In both samples, two regions (E2・C and E2・C-E5) of cDNAs were not amplified. Neither cDNA nor DNA of the E2 region was amplified in CIN-5. However, the E2 DNA from

Table 5 *HPV 16 mRNA expression and genome deletion in CINs*

case	cDNA				genomic DNA			
	E1 p16-11/12R	E2・C p16-16/17R	E2・C-E5 p16-18/19R	E7 p16-5/6R	E1 p16-11/12R	E2・C p16-16/17R	E2・C-E5 p16-18/19R	E7 p16-5/6R
CIN-5	+	−	−	++	++	−	−	+
CIN-6	+	−	−	++	++	+	+	+

CIN-6 was amplified by PCR.

DISCUSSION

Many experimental data have indicated etiological roles for specific HPV types in uterine cervical carcinoma and its precancerous lesions(1). In particular, the detection rate of HPV in cervical lesions is highly elevated by a modification of the PCR method(9-11). In contrast, the prevalence of HPV in normal cervices is relatively low(18).

There are many reports about the detection of HPV by PCR. Many types of primer sets such as type specific and universal primers have been reported(9, 10, 19-22). They are located in various regions of HPV, and the sensitivity and specificity are thought to be altered by the primer design.

On the other hand, the integration of HPV DNA into the human genome has been generally accepted as a characteristic of malignant lesions(23, 24). Conversely, episomal HPV 16 DNA has been found in cervical carcinoma tissues(2, 3). However, the techniques for the analysis of the physical state of HPV DNA in clinical specimens are time-consuming and require large amounts of template DNA, often unavailable for small premalignant CIN lesions.

One of the aims of this study was to determine the best position of primers to detect HPV DNA and to analyze the physical state of HPV 16 by PCR. We analyzed HPV 16 genome deletion by PCR using multi-primer sets and compared the results with those of two-dimensional electrophoresis. We previously established a detection method by using type-specific E6 primers and universal E7 primers(9, 10). They had high sensitivity and increased the detection rate for genital high-risk HPVs in cervical lesion when compared with the Southern blot method. However, there are many reports using different primers which are located in other regions of the HPV genome(20-22). Our data showed that genome deletion occurred in the E1, E2, E4, E5, L2 and L1 regions in 6 of our 20 samples. This is consistent with a previous report that the E6 and E7 ORFs are completely conserved and much of the non-coding region is free of base changes, including complete conservation of several regulatory elements(25). These

findings strongly suggest that the E6 and E7 regions are the most suitable regions to amplify the HPV DNA for its detection.

Donaldson *et al.* designed a novel, two-stage PCR assay to discriminate between integrated and episomal HPV 16 DNA(26). Another group utilized a pair of oligonucleotide primers specific to the E2 open reading frame, which is often deleted or disrupted following HPV integration, to distinguish between episomal and integrated forms by detecting amplification of the E2-specific fragment in the PCR product(27).

We found that, the E2 region was often deleted in integrated HPV 16. However, some deletions were seen even in the episomal HPV 16 DNA. The genome deletion shown by our PCR method was not consistent with the physical state analyzed by two-dimensional electrophoresis. It has been considered that two-dimensional electrophoresis is the most reliable method to discriminate between integrated and episomal HPV DNA. However, it seemed that genome deletion does not necessarily lead to the integration of viral DNA.

The second aim of this study was to analyze the expression of each mRNA in cervical lesions. We previously reported the expression of HPV 16 E6/E7 mRNAs and their splicing patterns in cervical lesions(5). E6/E7 mRNAs were expressed in all the HPV-positive cervical carcinomas and CINs. The E6 and E7 genes of HPV 16 directly participate in the immortalization of primary human keratinocytes(28), and they are consistently expressed in cervical carcinomas, CINs and cervical carcinoma cell lines(5, 29, 30).

HPV 16 and HPV 18 sequences contain splice donor and acceptor sites that potentially allow the synthesis of two short E6 proteins termed E6*(31, 32). These versions of E6 cannot be made in HPV 6 and HPV 11, and it is possible that E6* plays a role in malignant progression. It is reported that E6* is important for the efficient expression of the E7 product from the HPV 16 E6/E7 region (33).

The presence of the mRNA species previously described, which could encode the E2, E2C, E4, E5 and L2 proteins, was determined using our RT-PCR method. The sizes of the RT-PCR products were in agreement with previously mapped splice sites(30) and splicing sites were also confirmed by sequencing analysis. Products from the E2 gene region were implicated in the regulation of HPV gene transcription. E2 • C and the full-length E2 proteins are believed to act as trans repressors, tightly regulating the expression of the transforming E6 and E7 genes (34). We could not find any correlation between these genes.

The overall results of DNA and mRNA analyses in cervical lesions indicated that expression patterns of the early and late transcripts and the physical state or the deletion of the viral genome were not specifically related to the grade of

malignancy. Furthermore, alterations in the splicing patterns of HPV 16 transcripts may not be involved in tumor progression as described by Sherman et al (8). Thus, to elucidate the regulation of HPV 16 mRNA expression requires further investigation, including studies on cellular factors. Virus infection and viral gene expression emerge as necessary but apparently insufficient for cancer induction. Further modifications of host cells and viral genes appear to be required to cause malignant progression of infected cells.

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REFERENCES

(1) VOUSDEN KH. Human papillomaviruses and cervical carcinoma. Cancer Cells 1989, 1: 43-50.

(2) FUKUSHIMA M, YAMAKAWA Y, SHIMANO S, HASHIMOTO M, SAWADA Y, FUJINAGA K. The Physical State of Human Papillomavirus 16 DNA in Cervical Carcinoma and Cervical Intraepithelial Neoplasia. Cancer 1990, 66: 2155-2161.

(3) CULLEN AP, REID R, CAMPION M, LORINCZ AT. Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. J Virol 1991, 65: 606-12.

(4) TUREK LP. The structure function and regulation of papillomaviral genes in infection and cervical cancer. Adv Virus Res 1994, 44: 305-356.

(5) NISHIKAWA A, Shimada M, FUKUSHIMA M. Analysis of human papillomavirus type 16 E6/E7 mRNA in cervical cancers and precancerous lesions by means of the polymerase chain reaction with reverse transcriptase reaction. Acta Obst Gynaec Jpn 1991, 43: 1660-1666.

(6) CORNELISSEN MTE, SMITS HL, BRIET MA, van den TWEEL JG, STRUYK APHB, van der NOORDAA J, and ter SCHEGGET J. Uniformity of the splicing pattern of the E6/E7 transcripts in human papillomavirus type 16-transformed human fibroblasts, human cervical premalignant lesions and carcinomas. J Gen Virol 1990, 71: 1243-1246.

(7) JOHNSON MA, BLOMFIELD PI, BEVAN IS, WOODMAN CBJ, YOUNG LS. Analysis of human papillomavirus type 16 E6-E7 transcription in cervical carcinomas and normal cervical epithelium using the polymerase chain reaction. J Gen Virol. 1990, 71: 1473-1479.

(8) SHERMAN L, ALLOUL N, GOLAN I, DURSD M, BARAM A. Expression and splicing patterns of human papillomavirus type-16 mRNAs in pre-cancerous lesions and carcinomas of the cervix, in human keratinocytes immortalized by HPV 16, and in cell lines established from cervical cancers. Int J Cancer 1992, 50: 356-364.

(9) SHIMADA M, FUKUSHIMA M, MUKAI H, KATO I, NISHIKAWA A, FUJINAGA K.

Amplification and specific detection of transforming gene region of human papillomavirus 16, 18 and 33 in cervical carcinoma by means of the polymerase chain reaction. Jpn J Cancer Res 1990, 81 : 1-5.

(10) FUJINAGA Y, SHIMADA M, OKAZAWA K, FUKUSHIMA M, KATO I, FUJINAGA K. Simultaneous detection and typing of genital human papillomavirus DNA using the polymerase chain reaction. J Gen Virol 1991, 72 : 1039-1044.

(11) INOUE Y, YAMASHITA T, ISHIDA S, NISHIKAWA A, FUJINAGA Y, KUDO R, FUJINAGA K. Detection and typing of genital high-risk HPV DNAs in cervical scrapes using the E6E7-specific consensus PCR. Tumor Res 1995, 30 : 1-19.

(12) FUJINAGA Y, OKAZAWA K, NISHIKAWA A, YAMAKAWA Y, FUKUSHIMA M, KATO I, FUJINAGA K. Sequence Variation of Human Papillomavirus Type 16 E7 in Preinvasive and Invasive Cervical Neoplasias. Virus Genes 1994, 9 : 85-92.

(13) CHIRGWIN JM, PRZYBYLA AE, MACDONALD RJ, RUTTER WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 1979, 18 : 5294-5299.

(14) SEEDORF K, KRAMMER G, DURST M, SUHAI S, ROWEKAMP WG. Human Papillomavirus Type 16 DNA Sequence. Virology 1985, 145 : 181-185.

(15) MESSING J. New M13 vector for cloning. Methods Enzimol 1983, 101 : 20-78.

(16) SANGER G, NICKLEN S, COULSON AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 1977, 74 : 5463-5467.

(17) CHOW LT, NASSERI, M, WOLINSKY AM and BROKER TM. Human papillomavirus types 6 and 11 mRNAs from genital condylomata acuminata. J. Virol 1987, 61 : 2581-2588

(18) NISHIKAWA A, FUKUSHIMA M, SHIMADA M, YAMAKAWA Y, SHIMANO S, KATO I, FUJINAGA K : Relatively low prevalence of human papillomavirus 16, 18 and 33 DNA in the normal cervices of Japanese women shown by polymerase chain reaction. Jpn J Cancer Res 1991, 82 : 532-538.

(19) PAO CC, LIN CY, MAA JS, LAI CH, WU SY, SOONG YK. Detection of human papillomaviruses in cervicovaginal cells using polymerase chain reaction. J Infect Dis 1990, 161 : 113-115.

(20) PIZZIGHELLA S, PISONI G, BAVILACQUA F, VAONA A, PALU G. Simultaneous polymerase chain reaction detection and restriction typing for the diagnosis of human genital papillomavirus infection. J Virol Methods 1995, 55 : 245-256.

(21) SNIJDERS PJ, van, den, BRULE, Aj, SCHRIJNEMAKERS HF, SNOW G, MEIJER CJ, WALBOOMERS JM. The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J Gen Virol 1990, 71 : 173-181.

(22) YOSHIKAWA H, KAWANA T, KITAGAWA K, MIZUNO M, YOSHIKURA H, IWAMOTO A. Detection and typing of multiple genital human papillomaviruses by DNA amplification with consensus primers. Jpn J Cancer Res 1991, 82 : 524-531.

(23) DURST M, KLEINHEINZ A, HOTZ M, GISSMANN L. The Physical state of human papillomavirus in benign and malignant genital tumors. J Gen Virol 1985, 66 : 1515-1522.

(24) LEHN H, VILLA LL, MARZIONA F, HILGARTH M, HILLEMANS HG, SAUER G. Physical state and biological activity of human papillomavirus genomes in precancerous lesions of

femal genital tract. J Gen Virol 1988, 69 : 187-196.
(25) CONE RW, MINSON AC, SMITH MR, MCDOUGALL JK. Conservation of HPV-16 E6/E7 ORF sequences in a cervical carcinoma. J Med Virol 1992, 37 : 99-107.
(26) DONALDSON YK, ARENDS MJ, DUVALL E, BIRD CC. A PCR approach to discriminate between integrated and episomal HPV DNA in small clinical specimens. Mol Cell Probes 1993, 7 : 285-92.
(27) DAS BC, SHARMA JK, GOPALAKRISHNA V, LUTHRA UK. Analysis by polymerase chain reaction of the physical state of human papillomavirus type 16 DNA in cervical preneoplastic and neoplastic lesions. J Gen Virol 1992, 73 : 2327-2336.
(28) MURGER K, PHELPS WC, BUBB V, HOWLEY PM, SCHLEGEL R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol 1989, 63 : 4417-4421.
(29) SCHWARZ E, FREESE UK, GISSMANN L, MAWER W, ROGGENBUCK B, STREMLAU A, zur HAUSEN H. Structure and transcription of human papillomavirus sequence in cervical carcinoma cells. Nature 1985, 314 : 111-114.
(30) BAKER CC, PHELPS WC, LIDGREN V, BRAUN MJ, GONDA MA, HOWLEY PM. Structural and Transcriptional Analysis of Human Papillomavirus Type 16 Sequences in Cervical Carcinoma Cell Lines. J Virol 1987, 61 : 962-971.
(31) SMOKIN D, WETTSTEIN FO. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc Natl Acad Sci U S A 1986, 83 : 4680-4684.
(32) SCHNEIDER-GADICKE A, SCHWARZ E. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J 1986, 5 : 2285-2292.
(33) YAMADA T, YAMASHITA T, NISHIKAWA T, FUJIMOTO S and FUJINAGA K : Biologic activity of human papillomavirus type 16 E6/E7 cDNA clones isolated from SiHa cervical carcinoma cell line. Virus-Genes 10 : 15-25, 1995
(34) CRIPE TP, HAUGEN TH, TURK JP, TABARTBAI F, SCHMID PG, III, DURST M, GISSMAN L, ROMAN A, TUREK LP. Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-depandent enhancer, and by viral E2 trans-activator and repressor gene products : implications for cervical carcinogenesis. EMBO J 1987, 6 : 3745-3753.