A strong association between specific human papillomavirus (HPV) types and anogenital cancer has been well established. Certain types of HPV, including types 16, 18, 31, 33, 35, 45, 52, 56, and 58, play a pivotal role in the carcinogenesis of cervical cancer (33). In fact, the HPV viral DNA is identified in at least 90% of cervical carcinomas by PCR (2, 16, 30). The viral DNA is integrated into the cellular genome in cell lines derived from cervical carcinomas (3, 13, 27, 32) and in the majority of malignant tumors (6, 28). In contrast, integration of HPV DNA, regardless of type, occurs infrequently in preneoplastic-lesion, cervical intraepithelial neoplasia (CIN). Thus, integration has been proposed as an activation mechanism for progression from preneoplastic lesions to cervical cancers (2, 6, 28).

It is known that integration usually disrupts or deletes either the E1 or E2 open reading frame (ORF), which results in the loss of expression of the corresponding gene products. Disruption of the E1 and E2 genes also leads to overexpression of the E6 and E7 oncoproteins (11, 15), since the E2 gene product can repress activities from the HPV promoters that direct the expression of the E6 and E7 genes (1, 21, 26). The preferential disruption of the E2 gene will cause the absence of the E2 gene sequences in the PCR product following integration. Twenty-two carcinomas positive for HPV type 16 (HPV-16) DNA were first tested for the disruption of the E2 gene by PCR. A specific fragment of the E2 gene was not amplified in 10 cases, suggesting integration of HPV DNA into the host genome. Next, multiplex PCR for the HPV E2 and E6 genes was carried out in the remaining 12 cases. Copy numbers of both genes should be equivalent in episomal forms, while the E2 gene copy number will be smaller than that for E6 following the preferential disruption of the E2 gene in concomitant forms. Although relative ratios of HPV E2 to E6 PCR products (E2/E6 ratios) ranged from 1.40 to 2.34 in 10 of 12 cases, multiplex PCR products from 2 cases displayed extremely low ratios of 0.69 and 0.61. Southern blot hybridization with an HPV-16 probe revealed that only in these two cases was both episomal and integrated HPV DNA being carried simultaneously. Thus, multiplex PCR for the E2 and E6 genes of HPV-16 DNA following PCR for the E2 gene can distinguish the pure episomal form from a mixed form of episomal and integrated HPV DNA. Clinical application of this technique will help researchers to understand the implication of the integration of HPV DNA for cervical carcinogenesis and cervical cancer progression.

**Analysis by Multiplex PCR of the Physical Status of Human Papillomavirus Type 16 DNA in Cervical Cancers**

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Integration of human papillomavirus (HPV) DNA occurs early in cancer development and is an important event in malignant transformation of cervical cancer. Integration of HPVs preferentially disrupts or deletes the E2 open reading frame, which results in the loss of its expression. The preferential disruption of the E2 gene causes the absence of the E2 gene sequences in the PCR product following integration. Twenty-two carcinomas positive for HPV type 16 (HPV-16) DNA were first tested for the disruption of the E2 gene by PCR. A specific fragment of the E2 gene was not amplified in 10 cases, suggesting integration of HPV DNA into the host genome. Next, multiplex PCR for the HPV E2 and E6 genes was carried out in the remaining 12 cases. Copy numbers of both genes should be equivalent in episomal forms, while the E2 gene copy number will be smaller than that for E6 following the preferential disruption of the E2 gene in concomitant forms. Although relative ratios of HPV E2 to E6 PCR products (E2/E6 ratios) ranged from 1.40 to 2.34 in 10 of 12 cases, multiplex PCR products from 2 cases displayed extremely low ratios of 0.69 and 0.61. Southern blot hybridization with an HPV-16 probe revealed that only in these two cases was both episomal and integrated HPV DNA being carried simultaneously. Thus, multiplex PCR for the E2 and E6 genes of HPV-16 DNA following PCR for the E2 gene can distinguish the pure episomal form from a mixed form of episomal and integrated HPV DNA. Clinical application of this technique will help researchers to understand the implication of the integration of HPV DNA for cervical carcinogenesis and cervical cancer progression.

**MATERIALS AND METHODS**

Tissue specimens and DNA extraction. Primary lesions were screened for the presence of HPV DNA as described previously (17). Twenty-two invasive carcinomas specimens positive for HPV-16 DNA were included in the present study. Specimens were obtained at the time of admission for surgery at the Department of Obstetrics and Gynecology, Okayama University Medical School Hospital, Okayama, Japan. DNA was extracted from tissue specimens and CaSki cell lines (20) by a routine procedure of proteinase K digestion and phenol extraction.

**PCRs for E2 and a mixture of E2 and E6.** For detection of integration, the E2 ORF of the HPV-16 genome between nucleotides 2610 and 3836 was amplified according to the PCR conditions described by Park et al. (19). Next, multiplex PCR for the HPV E2 and E6 genes, both of which were in the same reaction tube, was performed. The primers for each sequence were 5'-CTTGGGACCACCAGAAACAC-3' (nucleotides 4388 to 4357) and 5'-TTGGTCACGTGCATTCCAG-3' (nucleotides 3770 to 3789) for the E2 gene and 5'-AAAGGCCTAAACGAAATCGGT-3' (nucleotides 26 to 46) and 5'-CATATACTCTACGCGCAG-3' (nucleotides 215 to 233) for the E6 gene. These primers yielded 352- and 208-bp fragments for the E2 and E6 sequences, respectively. The conditions for multiplex PCR were the same as those previously described for the c-erbB-2 gene and the mdm-2 genes (23, 24). All oligodeoxynucleotides were synthesized with a model 394 DNA synthesizer (Applied Biosystems, Foster City, Calif.). PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The UV-illuminated gels were photographed with Polaroid negatives (type 665), quantitated with an image scanner (GT8000; Epson, Suwa, Japan), and analyzed with Intelligent Quantifier software (Bio Image, Ann Arbor, Mich.).

**DISRUPTION OF THE E2 GENE.** The relative ratio of HPV E2 to E6 PCR products (E2/E6 ratio) was calculated. In order to verify the constancy of the E2/E6 ratio, the amount of template DNA (0.5 to 20 pg) or the number of amplification cycles (20 to 35) was altered by using HPV-16 plasmid DNA as the template DNA. E2/E6 ratios in this preliminary experiment always exceeded 1.28 (data not shown).

**Southern blot hybridization.** Ten micrometers of genomic DNA was digested with BamHI or PolII (New England Biolabs, Inc., Beverly, Mass.), electrophoresed in 1% agarose gels, and transferred onto nylon membranes (Hybond N; Amersham, Little Chalfont, Buckinghamshire, United Kingdom) by Southern blot procedures (25). BamHI has one cleavage site for HPV-16 DNA, while PolII is a multicut enzyme yielding the characteristic cleavage pattern. The membranes were sequentially hybridized with a 32P-labelled HPV-16 probe (11).
RESULTS

E2 PCR and multiplex PCR for E2 and E6. All invasive cervical cancer specimens were screened for the presence of HPV-16 DNA by nested PCR for the E6 gene (data not shown). Twenty-two carcinomas positive for HPV-16 DNA were then tested for the disruption of the HPV-16 E2 gene by PCR for E2. The specific fragment of the E2 gene (described above) was not amplified in 10 cases, suggesting integration of HPV DNA into the host genome in these cases. In contrast, the expected fragment of 1,027 bp was abundantly amplified in the remaining 12 cases (Fig. 1). In these cases, therefore, it was postulated that HPV DNA was present in episomal form without any disruption of the E2 gene. It was possible, however, that HPV DNA in these cancers exists in pure episomal form or in mixed episomal and integration forms.

Multiplex PCR for the HPV E2 and E6 genes was carried out in these 12 cases. Specific fragments of the E2 and E6 genes were successfully coamplified in all cases (Fig. 2). Although E2/E6 ratios ranged from 1.40 to 2.34 in 12 of 12 cases, multiplex PCR products from 2 cases exhibited extremely low ratios of 0.69 and 0.61.

Southern blot hybridization to analyze the physical status of HPV-16 DNA. Southern blot hybridization was carried out with an HPV-16 DNA probe for confident detection of the physical status of HPV-16 DNA in these cervical carcinomas. Ten micrograms of tumor DNA was first digested with single-cut enzyme BamHI and hybridized with an HPV-16 DNA probe. A single 7.9-kb band is supposed to appear when HPV DNA is episomal, and an off-sized fragment will appear when HPV DNA is integrated into the host genome (lanes 1). When the E2 gene was abundantly amplified, it was postulated that HPV DNA is present in episomal form without any disruption of the E2 gene (lanes 2, 3, 5, and 7).

DISCUSSION

The viral genomes are exclusively maintained as episomes in benign lesions induced by HPVs such as HPV-6 and -11 (10, 17). Only episomal HPV DNA is detected in CIN-I, and integrated sequences are rarely found in CIN-II and -III (6, 8). In contrast, the viral DNA is usually integrated into the cellular genome in cell lines derived from cervical carcinomas (3, 13, 27, 32) and in the majority of malignant tumors (6, 28). Thus, integration occurs early in cancer development and is an important event in malignant transformation (2, 6, 28). Several studies have also demonstrated the stable persistence of integrated HPV DNA in the invasive tumor cells or the presence of HPV DNA sequences in cancer cells that exist within metastatic lymph nodes (5, 7, 12, 18, 31). This phenomenon made it possible to predict the unexpected recurrence of cancer in metastatic lymph nodes.
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from the present study. It is widely believed that integrated sequences are rarely found in CIN-II and -III, but it is likely that the simultaneous presence of episomal and integrated forms in CINs and invasive carcinomas is more frequent. These lesions are generally small, and a sufficient amount of DNAs is not always available. A PCR-based analysis of the physical status of HPV DNA is applicable to small amounts of DNA purified from tiny lesions, formalin-fixed paraffin-embedded tissues, or cervical cytological specimens. Clinical application of this technique will help researchers to understand the implications of the integration of HPV for cervical carcinogenesis and the progression of cervical cancer.

Similar investigations for other types of HPV DNA will be easy to establish. In fact, we have found PCR primers that can coamplify the E2 and E6 genes of HPV-18 with satisfactory sensitivity and efficacy. Unfortunately, all carcinomas revealed the simultaneous presence of episomal and integrated forms in CINs and invasive carcinomas is more frequent. These findings indicate that the presence of both episomal and integrated forms of HPV is important in the progression of cervical cancer.

In conclusion, multiplex PCR for the E2 and E6 genes of HPV-16 DNA following PCR for the E2 gene can help researchers to analyze the physical status of HPV DNA.

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