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Genomic Aberrations of Human Papillomavirus Recovered from Cervical Cancers

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Human papillomaviruses (HPV) contribute to the development of malignancies of the uterine cervix and the viral E6 and E7 oncogenes are invariably retained and expressed in cervical cancer tissues. Minor, but not major, structural aberrations have been found quite frequently in viral DNA recovered from cervical cancer tissues. We examined the presence of the DNA sequence of HPV type 18 in 33 cervical cancer tissues by polymerase chain reaction. HPV type 18 DNA sequences was found in 24 of these 33 cervical cancer tissue specimens, and at least 21 of these 24 specimens did not appear to retain all the region and open reading frames examined. Twelve of these 24 tissues seemed to harbor only the E6 and/or E7 genes. These results can be construed to suggest that the absence of viral genes other than E6 and E7 is quite frequent in HPV recovered from cervical cancer tissues and that the E6 and E7 genes are important in the carcinogenesis of cervical carcinoma. It is possible that the E6 and/or E7 alone may be sufficient to maintain the transformed phenotype of cervical cancer.

Genital infection of certain types of human papillomavirus (HPV), including types 16 and 18, is associated with the development of cervical cancer (1, 2). Accumulative studies have suggested that two viral genes, the E6 and E7, are generally retained and expressed in cervical cancers (3, 4, 5). In cultured human keratinocytes, expression of the E6 and E7 genes of human papillomavirus types 16 and 18 can result in high-frequency immortalization of transfected cells. It has also been shown that transformation of primary human epithelial cells by HPV requires the combined effects of the E6 and E7 gene products, and that these two genes alone seem to be sufficient for the transformation (4, 5, 6, 7). Furthermore, the E6 and E7 proteins are capable of binding to the p53 and the retinoblastoma gene products *in vivo,* respectively, leading to the loss of the normal function of these tumor suppressor genes (8, 9, 10). All these observations point to the important and even essential roles the HPV E6 and E7 genes may play in the carcinogenesis of the uterine cervix.

Genome aberrations such as deletions, duplications and insertions have been found quite frequently in HPV DNA recovered from primary cervical cancer lesions and from cell lines derived from cervical cancers (11, 12, 13, 14). Most of these aberrations are small and occur in the E1-E2 open reading frames (ORFs) and in the upstream regulatory region (URR). To better our understandings of the presence of HPV DNA sequences and the state of viral genome aberrations in cervical cancers, we took to determine the frequencies of the presence of HPV type 18 viral DNA sequences in primary cervical cancer tissues.

MATERIALS AND METHODS

Materials. Cervical biopsy tissues were obtained from a total of 33 patients with cervical cancer. Guidelines for Human Experimentation of Chang Gung College of Medicine and Technology were followed. All tissue specimens were immediately frozen in liquid nitrogen after biopsy, and then processed to recover the total cellular DNA. Human cervical cancer cell line HeLa cells were obtained from American Type Culture Collection or ATCC (Rockville, MD) and were used as HPV type 18 DNA positive controls in the HPV amplification reactions.

Polymerase chain reaction (*PCR*) *amplification of HPV DNA.* Total cellular DNA was extracted from tissues by standard phenol-chloroform method and purified by alcohol precipitation before used for amplification. To achieve better sensitivity and specificity in the amplification and subsequent detection of HPV DNA, nested PCR that targeted various portions of the HPV 18 genomes were performed. Information on the primers and additional information have been described before

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(15, 16, 17). The conditions used for the amplification reactions have also been described earlier (15, 16, 17) with the following minor modifications. Briefly, 1–2 μg of purified cellular DNA was amplified with thermostable *Taq* DNA polymerase in a Thermal Cycler (Model 480, Perkin-Elmer Cetus, Norwalk, CT). The 50-μL amplification reaction mixture contained 10 mmol/L Tris HCl, pH 8.3; 50 mmol/L potassium chloride; 1.5 mmol/L magnesium chloride; 0.01% gelatin; 10 pmoles each of the primers for the initial amplification; 2.5 nmoles each of the four deoxyribonucleoside triphosphates, and 1 unit of *Taq* DNA polymerase (Perkin-Elmer Cetus). The temperature of the reaction mixture was cycled 32 times through 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C with a 10-min extension at 72°C at the end. For the nested PCR, 2 μL of the initial amplified reaction mixture were amplified following the same procedures in a freshly prepared 50-μL reaction mixture containing same components as those in the initial PCR and the new set of nested primers. Positive, negative and reagent controls were always included in each amplification reaction. The authenticity of amplified DNA was confirmed by both restriction endonuclease analysis (16, 17) and by Southern blot hybridization using internal oligonucleotides as probes (17, 18). A number of precautions were taken to minimize the possibility of contamination during sample processing and subsequent amplification reaction (19, 20, 21, 22). Repeated assays were performed on all specimens and all produced same results.

RESULTS

Figure 1 illustrates the amplification of the URR, and the L1, E6, E7 and E1 ORFs of HPV type 18. The sensitivities for the detection of these region and ORFs varied slightly and were such that between 100 and 300 viral copies per sample could be detected depending on the targets and primers used in the amplification reactions. These figures were determined by amplifying either serial dilutions of purified cloned HPV DNA of known concentrations or DNA prepared from HeLa cells (data not shown). Amplifications of 50 nanograms of human cellular DNA or 1 nanogram of bacterial *E. coli* DNA were always included in each assay as negative controls and always yielded negative results (data not shown).

The presence and distribution of the HPV type 18 DNA sequences that encode the URR region, and the L1, E6, E7 and E1 ORFs among the 33 cervical cancers are shown in Table I. Nine of the 33 cervical cancer specimens were found to be free of detectable HPV type 18 DNA sequences for the URR, and the L1, E6, E7 and E1 ORFs. HPV type 18 DNA from at least 21 of the remaining 24 specimens did not seem to harbor the entire HPV type 18 genome. Twelve of the 24 tissues that were positive for HPV type 18 DNA sequences appeared to contain only the E6 and/or E7 ORFs. The L1 ORF is the least prevalent region of HPV type 18 genome found in the cervical cancer tissues with a positive rate of only 21.2% (7 of out 33).

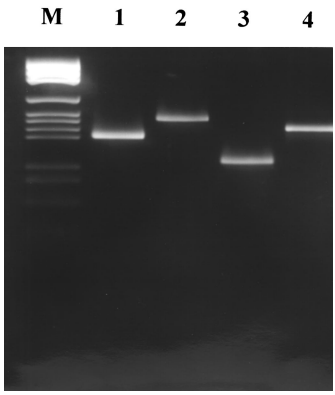

FIG. 1. Agarose gel electrophoresis of amplified HPV type 18 DNA. Plasmid pGEM-3 DNA digested with a mixture of 3 restriction endonucleases (*Hin*fI, *Rsa*I, and *Sin*I) was used as DNA size markers in lane M, and the sizes of these DNA fragments are (from top to bottom) 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51, and 36 and bp. Lanes 1, 2, 3, and 4 are amplifications of HPV type 18 URR, E6, E7, and E1 ORFs with nested primers, respectively. The sizes of the amplified HPV type 18 DNA in lanes 1 to 4 are 363, 477, 251, and 422 bp, respectively. Amplification of 50 ng of human cellular DNA or 1 ng of bacterial *E. coli* DNA with any of the primers yielded negative results (not shown).

DISCUSSION

The E6 and E7 genes of "high-risk" HPV types (most frequently types 16 and 18) have been documented to have transforming capability and termed HPV "oncogenes". Previous reports have indicated that combined effects of the E6 and E7 genes are necessary and may be sufficient to transform human primary keratinocytes (5), and that the E6 and E7 gene are almost always retained and expressed in cervical cancer tissues and in cell lines derived from cervical cancers (3, 4, 5). Our results presented in this study confirmed the presence of the E6 *or* E7 DNA sequences in all 24 cervical cancers that are positive for HPV type 18 DNA sequences. However, we have also found that substantial proportions of HPV type 18 DNA found in cervical cancers do not contain the entire genome. For example, HPV DNA recovered from only 3 cervical cancers contained all region and ORFs of HPV genome that were tested in this study (Table 1). DNA sequences encode the L1 ORF were only detected in 7 cervical cancers (Table 1).

Harboring of variant HPV, including deletion, insertions and duplications, in cervical cancer

TABLE 1
The Presence of HPV Type 18 Genes in CIN and Cervical Cancer Tissues

Case number	L1	URR	E6	E7	E1
7	+	+	+	+	+
9	+	+	+	+	+
11	+	+	+	+	+
4	+	+	+	+	−
13	+	+	+	+	−
8	−	+	+	+	+
10	−	+	+	+	+
33	−	+	+	+	+
5	−	+	+	+	−
6	+	+	−	+	+
17	+	+	−	+	−
1	−	+	−	+	−
14	−	−	+	+	−
15	−	−	+	+	−
16	−	−	+	+	−
12	−	−	+	−	−
2	−	−	−	+	−
3	−	−	−	+	−
18	−	−	−	+	−
19	−	−	−	+	−
20	−	−	−	+	−
21	−	−	−	+	−
22	−	−	−	+	−
23	−	−	−	+	−
26	−	−	−	−	−
27	−	−	−	−	−
24	−	−	−	−	−
25	−	−	−	−	−
28	−	−	−	−	−
29	−	−	−	−	−
30	−	−	−	−	−
31	−	−	−	−	−
32	−	−	−	−	−

Note. "+" denotes the presence and "−" the absence of DNA for respective genes.

tissues is not all that uncommon and has been reported before (11, 12, 13, 14, 23). However, most of these previously reported HPV genome aberrations involve only small regions of viral genome and have occurred mostly in the URR and the E1-E2 ORFs. Large deletions of HPV type 16 genome in cervical cancers have been cited in a recent report (24) in which a minimal conserved region of HPV type 16 viral integration of 2,745 base pairs (bp) in length, containing the entire URR and the E6 and E7 ORFs, has been proposed using PCR. A general deletion domain of 1,465 bp in the integrated HPV type 16 genome has been defined between 1417 nt to 2881 nt, covering most of the E1 ORF at the 3′-half and 60 bp at the 5′ terminus of the E2 ORF. Our findings of deletions in HPV type 18 were in general agreement with theirs except that we were able to find deletions in the L1, URR and even the E6 or E7 ORFs. The reasons for the difference is not clear at the present time.

We recognize that the determination of the presence or absence of certain DNA sequences like those reported in this study depends heavily on the detection limits or the amplification efficiencies. However, such difference in detection limits can not account for all the absence of certain HPV type 18 DNA sequences reported here, since the results presented in Table 1 have been calibrated to be based on more or less same detection limits for all HPV region and ORFs.

Increasing number of reports have appeared in the literature recently which used the consensus primers that targeted the E1 ORF (25) or the highly conserved L1 ORF (26, 27, 28, 29) for the screening and typing of multiple types of HPV DNA simultaneously. In view of the findings of others (24) and ours presented in this report that substantial proportions of HPV genomes found in the cervical cancer tissues may not comprise the E1 or L1 ORFs, the HPV prevalence data obtained based on the amplification of the E1 or L1 ORFs may thus result in either underestimation of HPV prevalence or even false-negative results (30, 31). Amplification reactions with primers that target the E6 or E7 genes, which is always retained in cervical cancers would therefore provide a more accurate assessment of HPV prevalence. It remains unclear what is the mechanism through which large portions of HPV genome can be deleted. Works are currently underway to clone the integration junctions and sequencing of the HPV as well as flanking host DNA sequences, which hopefully will reveal to us whether there is universal or consensus sequences at these junctions.

In summary, our results seem to suggest that HPV type 18 genomes found in cervical cancers frequently do not contain the entire viral genome. These findings suggest that genome aberrations such as deletions are a common feature in HPV found in cervical cancers. The fact that we could always detect the E6 and/or E7 genes further underscores the functional importance of the E6 and E7 genes which may be sufficient to maintain the transformed phenotype of cervical epithelial cells.

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