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Integration of human papillomavirus types 16 and 18 in cervical epithelial lesions using tyramide-amplified *in situ* hybridization

Ei Kawahara, Miwa Akasofu Imai*, Tomohiro Mizutani
Tamiko Umei, Tomokatsu Kanitani, Yuzuru Takeuchi**

Abstract

Highly sensitive *in situ* hybridization (ISH) and highly sensitive PCR methods were used to determine the infection of HPV type 16 (HPV 16) and type 18 (HPV 18) and the integration of them into the cells of squamous and glandular lesions of the uterine cervix. The frequency of HPV infection detected by ISH and PCR increased with progression of squamous lesions. All cases of ICC showed the integrated pattern of positive signals with ISH and all positive cases in CIN1 showed the episomal pattern. In CIN2 episomal patterns were more frequent than integrated pattern and in CIN3 integrated pattern were more frequent. Glandular lesions showed a greater frequency of HPV 18 than HPV 16 on nested PCR and ISH, and almost all of ISH-positive cases showed the integrated pattern. These data suggested that integration of HPV 16 and 18 is required for carcinogenesis in both squamous cell carcinoma and adenocarcinoma of the uterine cervix.

Key words

Cervical carcinoma, human papillomavirus, in situ hybridization, nested PCR, viral integration

Introduction

Large-scale analyses of invasive squamous cell carcinoma (ICC) and cervical intraepithelial neoplasia (CIN) grade 2 and 3 have demonstrated that nearly all of these lesions contain human papillomavirus (HPV) DNA.[1,2] Many studies have also indicated that cervical carcinoma and the precursor lesions are associated with more than 10 types of high-risk (HR) HPV.[3,4] Nevertheless, the majority of transient infections disappear without any lesion in cervix.[5,6] Furthermore, showing regression in many cases of low grade CIN, persistence was found in 30% with progression to CIN III or to invasive carcinoma in less than 10%.[7,8] Thus, detection of HR HPV, especially in combination with routine Pap smears, should be a useful means screening cases with possible severe outcome, although novel biomarkers which predict more precise prognosis are required to detect lesions that have a high risk of development to ICC.[9]

Integration of HR HPV into the genomes of host cells has been suggested to represent a malignant phenotype. In low-grade CIN, HPV genomes persist as episomal molecules, while viral genomes are frequently integrated into the genomes of cancer cells.[10-13] The development of malignant cells seems to be closely linked to integrated HR HPV. However, the prevalence and significance of HPV integration among cervical lesions of different grades is still uncertain, and the incidence in low-grade CIN has not been examined in detail.

* Department of Clinical Laboratory Science, Kanazawa University Graduate School of Medical Science
** Department of Health Science, Ishikawa Prefectural Nursing University
Division of Gynecology, Fukui General Hospital, Japan

In situ hybridization (ISH) has been used for detection of integrated HPV DNA using formalin-fixed and paraffin-embedded (FFPE) thin sections. The assessment of punctate signals as integration and diffuse pattern as episomes with ISH seems to be established.[14-15] In the present study, we examined infection by HPV 16 and 18 with tyramide-amplified ISH to determine how frequently HPV genomic DNA is present and integrated into the genomes of the cells of squamous and glandular lesions of the uterine cervix.

MATERIALS AND METHODS

1. Tissue samples

Tissues obtained by biopsy, conization, or hysterectomy were fixed with 10% neutral buffered formalin and embedded in paraffin. Ethics approval for the study was obtained from the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medicine and the Ethics Committee at Fukui General Hospital.

2. Virus detection by nested PCR

DNA was extracted from one section of FFPE tissues as described before.[3] The DNA was added to a standard PCR mixture. PCR was performed using KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan). Primer sets for HPV 16 and 18 were designed to amplify DNA containing sequences of the respective E6 region. Sequence data from GenBank (accession number HPU89348 for HPV 16 and HPU89349 for HPV 18) were used to design the primers. Nested PCR was applied to all samples using the forward primer and the second reverse primer. To verify sufficient extraction of DNA from FFPE tissues, extracts were also processed in parallel for amplification of the human β-globin gene.

3. Virus detection by tyramide-amplified ISH.

The GenPoint System (DAKO, Tokyo, Japan) using the tyramide amplification method was used for in situ hybridization according to the manufacturer's protocol. Background quenching in all specimens was performed with 0.3% H_2O_2, 0.1% avidin, and 0.01% biotin. Biotin-labeled specific probes for HPV 16 (Y1407; DAKO) and 18 (Y1408; DAKO) were applied to separate sections. For positive and negative controls, control slides with HPV-infected SiHa cell line, a biotin-labeled positive control probe specific for HPV 16, and a biotin-labeled negative control probe for plasmid DNA were used. Cytoplasmic diffuse dots were considered to be due to nonspecific reaction. When almost all nuclei were diffusely positive without cytoplasmic positivity, mimicking a diffuse pattern, but normal epithelia and lymphocytes in the proximity of the lesion showed similar diffuse reaction, the reaction was judged to be nonspecific.

4. Statistical analysis

The differences between HPV-positive cases and HPV-negative cases, or punctate cases and non-punctate cases among different grades of squamous lesions were analyzed by Fisher's exact probability test.

RESULTS

All the bands of PCR products using present primers at 115 bp for HPV16 and 119 bp for HPV18 were cut out of the agarose gels, extracted, and sequenced. All of them were confirmed to be the sequence of HPV. The results with nested PCR showed that the frequency of HPV infection increased with progression of squamous lesions, eventually reaching 100% in ICC. Statistically there was a significant difference ($P<0.05$) between low grade squamous lesions (CIN1 and CIN2) and high grade squamous lesions (CIN3 and ICC). Nested PCR detected HPV 18 frequently in CIN3 (46%) and ICC (63%) although the frequencies were lower than those of HPV 16 (Table 1).

ISH also showed an increase in frequency of HPV 16 infection with increasing grade, and the frequency reached 100% in ICC (Table 1). The frequency of positivity for HPV 18 with ISH was lower than that with single PCR and higher than that with nested PCR, but in some cases HPV was detected with ISH despite the lack of detection with nested PCR (Table 1). Positive signals were observed as a punctate pattern, diffuse pattern, or a mixed punctate and diffuse pattern. All the positive cases of ICC showed punctate signals although one showed the mixed pattern, and 5 of

Table 1. Percentages of positivity or pattern detected by each method. ISH, *in situ* hybridization; n, number of cases; CIN, cervical intraepithelial lesion; ICC, squamous cell carcinoma; AC, adenocarcinoma; AIS, adenocarcinoma *in situ*.

		ISH (%) diffuse/mixed/punctate		PCR (%)		ISH or PCR (%)	
lesion	(n)	16	18	16	18	16 or 18	16 & 18
CIN1	(10)	20/0/0	20/0/0	30	30	60	10
CIN2	(6)	14/0/14	0/0/14	29	57	70	14
CIN3	(13)	7/30/23	23/7/15	85	85	100	77
ICC	(8)	0/0/100	0/13/50	75	100	100	75
AIS+AC	(25)	0/0/25	0/13/50	88	100	100	30

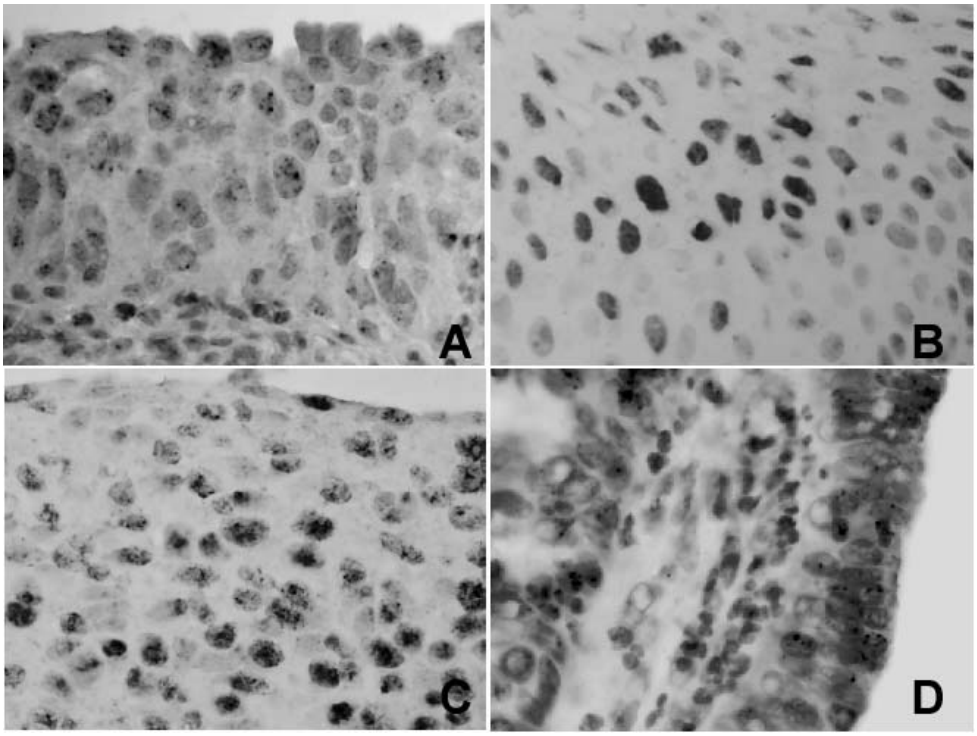

Fig. 1. Photomicrographs of in situ hybridization for HPV 16 and 18. A. The punctate pattern of HPV 18 in CIN3. B. The diffuse pattern of HPV 18 in CIN2. E. The punctate pattern of HPV 16 in adenocarcinoma in situ. C. The mixed pattern of HPV 18 in CIN3. D. The punctate pattern of HPV 18 in adenocarcinoma.

11 HPV 16- or 18-positive CIN3 cases showed pure punctate pattern, 4 cases mixed, and only 2 cases showed a diffuse pattern (Table 1). The pattern of positive signals was characteristically diffuse in all CIN1 ISH-positive cases, while all ICC cases had a punctate pattern. A pure punctate pattern was observed in only 1 CIN2 case (13%). The punctate patterns were found with a significant difference in high grade squamous lesions in comparison to low grade squamous lesions ($P<0.05$).

HPV 16 or 18 was detected in all ICC cases examined with both methods. Mixed infection was also marked in ICC as well as in CIN3.

Invasive adenocarcinoma and adenocarcinoma *in situ* were also examined. HPV 18 was detected with ISH and nested PCR in AC and AIS lesions as frequently as in CIN3 and ICC, and much more frequently than HPV 16 in AC and AIS (Table 1). Among HPV 16- or HPV 18-positive cases on ISH, all but one had a punctate pattern. All cases of CIN3, ICC, and AC were positive for HPV 16 or 18 on ISH or nested PCR.

DISCUSSION

HPV infection detected with tyramide ISH and single PCR was as frequent as reported in a previous study by Dabic *et al.*[16] using similar

methods. They concluded that combined examination with ISH and PCR methods is required to avoid overestimation of the background signal mimicking a diffuse pattern with ISH. By combined examination with ISH and PCR, HPV 16 or 18 was eventually detected at rates of 100% in CIN3, ICC, and AC, and thus HPV 16 or 18 could be considered to play a crucial role in carcinogenesis of the uterine cervix. In addition, the frequencies of HPV 18 infection in the present study, 75% in ICC and 85% in CIN3, second to HPV 16, were higher than in previous reports.[1] The primers used in the present experiments were specific because all the PCR products of CIN and ISS cases were sequenced and clarified the sequence of HPV. The probes for ISH, which are commercially available, have been used equivalently, and there were no report of cross-reaction. The prevalence of HPV 18 detected here was probably due to the high sensitivity of nested PCR and tyramide-amplified PCR methods.

It has been reported that the diffuse signal pattern suggests episomal HPV, while the punctate pattern suggests integration of HPV into the genome of the host cell.[15] The present data were consistent with several previous studies in that all ICC cases had the punctate pattern. All CIN1 cases were reported to have the diffuse pattern with no cases showing the punctate pattern[3,10] except in a study by Cooper *et al.*[15] who reported rates of 49% in CIN1, 64% in CIN2, and 83% in CIN3. The frequency of positivity in CIN2 and CIN3 using ISH varied from 10–50%.[12,14,15] In the present study, there was a marked difference in the frequency of the punctate pattern in CIN2 and CIN3, 13% and 77%, respectively. Nevertheless, previous reports have indicated that the punctate signal in such intraepithelial lesions is a predictor of poorer prognosis.[12,15] Recent findings suggested that the pure integrated pattern and the mixed episomal and integrated pattern show biological differences. Evans *et al.*[17] reported that low- and high-grade CIN with the mixed integrated and episomal HPV regressed spontaneously. They suggested that episomal HPV inhibited the expression of E6 and E7 genes of integrated HR HPV due to the presence of the E2 gene in episomal HPV despite the lack of an E2 gene in integrated HPV, which subsequently inhibits the proliferation of HPV-integrated cells. Thus, the punctate pattern with disruption of the E2 gene and hence the absence of E2 gene expression rather than the mixed pattern may be a marker for high likelihood of progression to invasive carcinoma. The integrated HPV genes may shorten the duration of malignant transformation.[18] Assuming this is correct, one of 8 CIN2 cases and 5 of 13 CIN3 cases in the present study should be considered at risk of progressing to carcinoma.

Adenocarcinoma in situ and invasive adenocarcinoma were associated more frequently with HPV 18 than HPV 16, as shown in many previous studies.[19] These results suggested that HPV 18 is the main risk factor for the development of adenocarcinoma, whereas HPV 16 is associated with both ICC and adenocarcinoma. The results of the present study clarified further that HPV 16 or 18 were integrated in cells of invasive adenocarcinoma and adenocarcinoma in situ in all cases in which HPV was detected. Mixed infection was found less markedly than those of high grade squamous lesions.

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In situハイブリダイゼーションによる子宮頸部上皮性病変への16, 18型ヒト乳頭種ウイルスの組み込みの検出

河原　栄, 今井　美和*, 水谷　智洋, 梅井　民子, 蟹谷　智勝, 竹内　譲**

要　　旨

高感度in situハイブリゼーション（ISH）法と高感度PCR法を用いて子宮頸部扁平上皮病変と腺病変での16型と18型のヒト乳頭種ウイルス感染頻度検索し、核への組み込みの頻度をISH法で決定した。16, 18型はPCRとISHでともに、扁平上皮系病変のグレードが高まるにつれて感染頻度が上がった。浸潤扁平上皮癌のすべての例でISHで組み込み型を示し、CIN1の全てでエピソーム型を示した。腺系病変ではPCRでもISHでも16型より18型が多く検出され、そのほぼすべてが組み込み型だった。CIN2ではエピソーム型が多くCIN3では組み込み型が多かった。これらから16, 18型の核への組み込みは扁平上皮病変でも腺病変でも癌の初期に現れる変化、あるいは癌への進展を強く示唆する所見と考えられた。