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Original contribution

Human papillomavirus multiplex ligation-dependent probe amplification assay for the assessment of viral load, integration, and gain of telomerase-related genes in cervical malignancies☆

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Summary We evaluated the reliability of a novel multiplex ligation-dependent probe amplification (MLPA) assay in detecting integration of human papillomavirus (HPV) based on the viral *E2*/*E6* copy number ratio in formalin-fixed and paraffin-embedded cervical lesions. The MLPA results were compared with those of amplification of papillomavirus oncogene transcripts for RNA, detection of integrated papillomavirus sequences for DNA, and HPV fluorescence in situ hybridization (FISH). DNA was isolated from 41 formalin-fixed and paraffin-embedded HPV-positive cervical lesions (cervical intraepithelial neoplasia grade 3 lesions, squamous cell carcinomas, and adenocarcinomas) for MLPA analysis. From 13 matching frozen samples, DNA and RNA were isolated for the detection of integrated papillomavirus sequences and/or the amplification of papillomavirus oncogene transcripts, respectively. Integrated HPV16, HPV18, or both were identified. The MLPA assay detected viral

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integration in 12 of these 13 cases, and episomal copies also were detected in 7 cases. In 20 of the 24 cases with exclusive viral integration or episomal viral copies as detected by FISH, MLPA confirmed the physical status of the virus. In the cases classified as mixed by FISH, the presence of excess episomal copies complicated the recognition of viral integration by MLPA. Furthermore, the feasibility of detecting gain of the telomerase genes with the HPV MLPA assay was evaluated. The MLPA confirmed the FISH data in 12 of 13 cases in which the status of copy number gain for telomerase RNA component was known. In conclusion, the HPV MLPA assay can be performed on routinely processed cervical lesions for the detection of viral load and HPV integration.

1. Introduction

Uterine cervical cancer is the second most common cancer in women worldwide, with approximately 500 000 new cases and 275 000 deaths estimated in 2002 [1]. Human papillomavirus (HPV) plays a causal role in the development of this disease [2] and has been identified in most cervical carcinomas [3]. In addition to cervical carcinoma, HPV has been identified in several other sites, including tonsillar [4], anal [5], and penile [6] carcinomas. HPV types 16 and 18 are the most prevalent oncogenic types in cervical carcinoma and are responsible for more than 70% of cases [7,8].

However, although HPV infection is an indispensable factor, it is not sufficient to cause cancer [9]. For HPV16, a high viral load is described as an indicator of persistent infection, which, in turn, is associated with progression to carcinoma. Furthermore, integration of the HPV genome into the host genome is associated with progression to carcinoma [10]. Typically, part of the *E2* gene and sometimes also the *E1* or *L1* genes [11] are lost on integration into the host genome, whereas *E6* and *E7* almost always are retained. Several studies that have analyzed the most frequently deleted region were not able to identify 1 general minimum region that is always deleted [12,13].

One way to analyze integration is by the detection of viral cellular fusion transcripts, as is done with the amplification of papillomavirus oncogene transcripts (APOT) assay [14]. Because the presence of human sequences in the transcript is used as the indicator of integration, the APOT assay is able to detect all actively transcribed cases with integration independent of the location of the viral deletion. Unfortunately, the need for RNA makes it undesirable to use this assay for analysis of routinely processed formalin-fixed and paraffin-embedded (FFPE) samples, as it is difficult to retrieve good-quality RNA from this type of sample [15].

The detection of integrated papillomavirus sequences (DIPS) assay is a DNA assay that also uses the fusion between viral and human sequences for the detection of integration [16]. However, most amplified products have fragments larger than 300 base pairs, making it difficult to apply this assay to FFPE tissue samples. Alternative assays that can be used to determine integration in paraffin-embedded tissue are described, but their sensitivity in detecting integration is limited. Fluorescence in situ hybridization (FISH) distinguishes episomal from integrated HPV based on a diffuse or punctate FISH pattern, respectively. However, the interpretation of these patterns is subjective, and there can be both interobserver and intraobserver differences. Other integration assays use the predominant deletion of the *E2* gene for the analysis of viral integration through amplification or lack of amplification of an *E2* fragment [17-19], but this assay will not detect integrated HPV in the presence of episomal copies. On the other hand, by determining the copy number ratio between *E6*, which is never deleted, and *E2*, integration is measured even in a background of episomal copies based on the relative loss of *E2* compared with *E6* [18]. Although the interpretation of these results is more objective, the *E2* amplification usually focuses on a specific region within the *E2* gene, and integration as a result of deletions outside this region will be missed.

Chromosomal aberrations also correlate with progression to cancer. These changes can be analyzed by FISH and by quantitative polymerase chain reaction (PCR) and array comparative genomic hybridization. However, the latter 2 are difficult to apply to FFPE tissue because of the presence of significant numbers of normal cells in each sample. Furthermore, high-quality DNA is needed to perform array comparative genomic hybridization, and this is difficult to obtain from paraffin-embedded material.

Recently, we developed a multiplex ligation-dependent probe amplification (MLPA) assay to detect HPV16/18 simultaneously and to quantify viral load, viral integration, and gain of the telomerase genes. The MLPA is a molecular technique initially developed by Schouten et al [20] for the quantification of as many as 40 genomic targets. For each target, a pair of probes is designed, each of which contains a universal PCR primer sequence and a sequence complementary to the target. When the probes hybridize immediately adjacent to each other, they can be ligated and subsequently amplified using universal primers. Because one of the primers is labeled with a fluorescent dye, the amplified products can be seen using capillary electrophoresis. Furthermore, the products can be discerned based on length because of the variable stuffer sequences [21]. We modified the assay such that a simultaneous quantification of both human and viral targets is possible. The HPV MLPA assay was tested on model systems and on fresh frozen samples of normal cervix, cervical intraepithelial neoplasia (CIN), and cervical cancer as well as cytologic samples [22].

The aim of this study was to determine whether the assay also is applicable to simultaneous analysis of viral load and

integration and gain of the telomerase genes in FFPE tissue samples. For this study, the MLPA assay was initially performed on a series of FISH samples verified for the physical status of the virus [23]. The status of 3q copy number gain was known for several of these cases. In a second series of FFPE tissue samples, the APOT and DIPS assays had confirmed viral integration in the matching fresh frozen samples [24]. In addition, FISH was performed on this latter series to confirm the MLPA or APOT results or both.

2. Materials and methods

2.1. Materials

FFPE tissue sections from 33 invasive carcinomas (24 squamous cell and 9 adenocarcinomas) and 8 high-grade squamous intraepithelial lesions were selected for this study (Tables 1 and 2). For 13 cases, matching paraffin-embedded and fresh frozen tissue was available (series A). For these cases, paraffin-embedded sections were used for FISH analysis, and DNA was isolated from small parts of these sections, representing both normal and tumor tissue, through microdissection. Microdissection of 4-μm sections was performed under an inverted microscope using the edge of a 150-μm-thick coverslip, guided by a p16-immunostained parallel section. The p16 immunostaining as a surrogate marker for HPV infection was performed as previously described [25]. The estimated proportion of tumor cells exceeded 70%. For 10 of these 13 cases, 2 or 3 sections of the same lesion were available. The matching fresh frozen tissue was used for the isolation of both RNA and DNA for APOT and DIPS analysis, respectively. For the 28 remaining cases (series B), only paraffin-embedded tissue or FFPE sections were available. For these as well as for 7 normal cervical tissues used as reference, DNA was isolated from tissue microdissected as described (series A) or from three 10-μm whole tissue sections without microdissection (series B and reference samples) using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2. Multiplex ligation-dependent probe amplification assay

The MLPA assay was performed as described [19] using reference samples containing episomal HPV16 or 18 with a viral load of 2 copies per cell in a background of normal human DNA in each experiment to correct for experimental variability. Peak height data were exported to Excel files for calculation of ratios and analyzed. The HPV positivity was determined based on the presence of both *E6* and *E7* peaks. The *E6* and *E7* loads were estimated by determining the signal intensity ratio between *E6* or *E7* and 7 human targets, that is, *β-globin* (2×), MutS homolog 2, telomerase reverse transcriptase (*TERT*) (2×), and telomerase RNA component (*TERC*) (2×). The average load of *E6* and *E7* was multiplied by 2 to obtain the viral load in a diploid cell. For the detection of the physical status, 2 *E2* probes (*E2.1* and *E2.2*) per HPV type were applied [19], which target the sequences most frequently deleted on integration into the human genome. The ratios for each sample were compared with the ratios for the reference samples containing episomal HPV16 or HPV18 to determine gain of *TERT* and *TERC* as well as the percentage of integration. For the 13 cervical cancer samples with a matching (microdissected) normal sample, the latter was used as the reference to determine gain of *TERT* and *TERC*. Because of the variability within the measurements, integration of less than 30% of HPV copies was classified as

Table 1 Summary of APOT, DIPS, FISH, and MLPA results in series A

Case	APOT		DIPS	FISH	MLPA			Histologic classification
	HPV type	Physical status	Viral disruption site	Physical status	HPV type	Viral load[a]	Physical status	
1	16	Int	2498	Int	16	1.6	Int	SCC
2	18	Int	2241	Inconcl	18	1.1	Int	SCC
3	16; 18	Int	2048	Inconcl	18	1.3	Int	SCC
4	18	Int	2343	Inconcl	18	0.6	Int	SCC
5	18	Int	2946	Int	18	2.1	Int	AdCa
6	16	Int	3438	Int	16	0.8	Mix	SCC
7	16	Int	>3959	Int	16	30.9	Mix	SCC
8	18	Int	2466	Int	18	5	Mix	AdCa
9	16	Int	–	Inconcl	16	1	Epi	SCC
10	16	Mix	1654	Int	16	32.5	Mix	SCC
11	16	Mix	1200	Int	16	30.9	Mix	SCC
12	18	Mix	>2438	Inconcl	18	0.9	Mix	AdCa
13	18	Mix	1733	Int	18	12.2	Mix	AdCa

NOTE. For this series, microdissected tissue sections were used for DNA isolation as described in the "Materials and methods" section.
Abbreviations: AdCa, adenocarcinoma; Epi, episomal; Inconcl, inconclusive; Int, integration; Mix, mixed combined integrated and episomal viral copies; SCC, squamous cell carcinoma.
[a] Viral load is reported as the copy number per genome.

Table 2 Summary of the HPV-FISH and MLPA results in series B

Case	HPV type	FISH physical status	MLPA				Histologic classification
			Viral load HPV16	Viral load HPV 18	Physical status	High-risk classification	
1	18	Int		0.3 ± 0.1	Int	+	AdCa
2	16; 18	Int	2.2 ± 0.6	0.2 ± 0.1	Mix; Int	+	SCC
3	16; 18	Int	0.2 ± 0.1	0.2 ± 0.1	Mix; Mix	+	AdCa
4	16	Int	9.0 ± 1.6		Mix	+	SCC
5	18	Int		2.0 ± 0.8	Mix	+	CIN3
6	18	Int		1.8 ± 0.8	Mix	+	CIN3
7	18	Int		0.2 ± 0.1	Mix	+	AdCa
8	16; 18	Int	0.1 ± 0.1	1.5 ± 0.4	Epi; Mix	+	CIN3
9	16	Int	9.9 ± 1.0		Epi	−	AdCa
10	16	Int	5.5 ± 0.6		Epi	−	SCC
11	16	Int	4.5 ± 0.6		Epi	−	SCC
12	16	Int	1.3 ± 0.8		Epi	−	CIN3
13	16; 18	Mix	5.7 ± 0.5	<0.05	Epi; Epi	−	CIN3
14	16; 18	Mix	207.3 ± 63.6	0.1 ± 0.1	Epi; Epi	+	SCC
15	16; 18	Mix	4.4 ± 1.2	0.2 ± 0.1	Epi; Epi	−	SCC
16	16; 18	Mix	71.9 ± 22.1	0.5 ± 0.2	Epi; Epi	+	SCC
17	16; 18	Mix	111.5 ± 31.0	<0.05	Epi; Epi	+	SCC
18	16	Mix	47.9 ± 3.6		Epi	+	CIN3
19	16	Mix	63.9 ± 0.9		Epi	+	AdCa
20	16	Mix	4.5 ± 1.8		Epi	−	SCC
21	16	Mix	40.0 ± 7.8		Epi	+	SCC
22	16	Mix	196.9 ± 28.5		Epi	+	SCC
23	16; 18	Epi	183.8 ± 60.0	0.1 ± 0.1	Epi; Epi	+	SCC
24	16	Epi	2.7 ± 0.8		Epi	−	CIN3
25	16	Epi	25.7 ± 3.3		Epi	+	CIN3
26	16	Epi	20.8 ± 1.0		Epi	−	SCC
27	16	Inconcl	0.1 ± 0.1		Epi	−	SCC
28	16; 18	Inconcl	1.7 ± 0.5	<0.05	Mix; Inconcl	+	SCC

NOTE. For this series, whole tissue sections were used for DNA isolation, as described in the "Materials and methods" section.
Abbreviations: CIN3, cervical intraepithelial neoplasia grade 3; Epi, episomal; Inconcl, inconclusive; Int, integration; Mix, mixed combined integrated and episomal viral copies; high-risk classification, integration or mixed HPV status and/or viral load greater than 25 copies per genome; viral load, mean copy number per genome ± SD.

(predominantly) episomal; between 30% and 95% of integrated virus was classified as mixed (ie, episomal and integrated) HPV, and greater than 95% of integrated virus, as integrated HPV.

All whole tissue samples were analyzed in at least duplicate. The microdissected samples could be analyzed only once.

2.3. Fluorescence in situ hybridization

The FISH analysis was performed using digoxigenin (Dig)–labeled HPV16 and HPV18 probes (PanPath, Uden, The Netherlands) in the mild and harsh pretreatment protocols [26]. The probes were detected using the tyramide signal amplification procedure as previously described for single-target hybridization using rhodamine-labeled tyramide [27]. In short, the Dig-labeled probe was detected by peroxidase-conjugated sheep antidigoxigenin Fab fragments (SHaDIG, 1:100; Roche Molecular Chemicals, Basel, Switzerland) or first mouse antidigoxigenin (MaDig, 1:2000; Sigma Chemical Co, St Louis, MO), then a peroxidase-conjugated rabbit antimouse (RaM, 1:100; DAKO A/S Glostrup, Denmark), and finally a peroxidase-conjugated swine antirabbit (SwaR, 1:100; DAKO) all for 30-minute incubations at 37°C and washed in phosphate-buffered saline (PBS)/0.05% Tween-20. Thereafter, the tyramide signal amplification reaction was carried out under a coverslip by applying 50 μL (1:500 diluted from a 1 mg/mL stock solution in ethanol) rhodamine-labeled tyramide in PBS containing 0.1 M imidazole, pH 7.6, and 0.001% H_2O_2 for 10 minutes at 37°C. The slides were washed in PBS containing 0.05% Tween-20 (Janssen Chimica, Beerse, Belgium), dehydrated in an ascending ethanol series and mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole (0.5 ng/μL; Sigma Chemical Co).

Images were recorded with the Metasystems Image Pro System (black and white CCD camera; Sandhausen, Germany) mounted on a Leica DM-RE fluorescence microscope equipped with tetramethylrhodamine isothiocyanate (TRITC) and 4′,6-diamidino-2-phenylindole single bandpass filters for single-color analysis.

Controls included HPV16 and HPV18 hybridization on tissue sections of FFPE HPV-positive cell lines (SiHa,

CaSki, and HeLa). Signal morphology was categorized as follows. When nuclei were completely and homogeneously stained, the signal was classified as episomal, whereas discrete nuclear signals (1-3 per nucleus) in a clean background were classified as integrated, in line with the criteria of Cooper et al [28].

The FISH patterns were classified by A. H. and A. H. N. H. Discordant interpretations were resolved by review of the samples until consensus was reached. Both A. H. and A. H. N. H. were blinded to the outcomes of the MLPA, APOT, and DIPS assays. The histologic diagnosis was known to both.

2.4. Amplification of papillomavirus oncogene transcripts

Total RNA was reverse transcriptase transcribed, and the resulting HPV oncogene transcripts were amplified as described [14], with minor modifications. Total RNA (500-1000 ng) was transcribed using 100 U of MMLV reverse transcriptase Superscript II (Invitrogen Life Technologies, Carlsbad, CA) and an adaptor-linked oligo(dT) primer [29]. Subsequent seminested PCR was performed using HPV *E7*-specific forward primers and reverse adaptor primer and an oligo(dT) primer, respectively.

2.5. Detection of integrated papillomavirus sequences

The DIPS assay was performed as described previously [16], with minor modifications. Digestion of 1.2 μg of genomic DNA by restriction endonuclease Sau3AI was followed by ligation of an enzyme-specific double-stranded adaptor containing a double- and a single-stranded part ("vectorette feature"). After the first linear PCR using distinct viral primers for HPV16 and HPV18, a second PCR step using a nested viral primer and an adaptor primer 1, which is complementary to the missing strand of the single-stranded part of the adaptor, was performed.

2.6. Sequence analysis of viral cellular junctions obtained by APOT and DIPS

The PCR products of interest were excised from the gel and extracted using the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing reactions were performed using the Big-Dye terminator DNA-sequencing kit (Perkin Elmer, Boston, MA) and an ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, CA). Sequencing results were analyzed using the BLASTN program provided by the US National Cancer Institute.

3. Results

Viral type, load, and integration as well as gain of the telomerase genes have been shown to be useful in determining progression to cervical cancer. This has been demonstrated using the HPV MLPA assay on fresh frozen tissue samples as well as by other groups of investigators using other assays, including APOT, DIPS, FISH, and quantitative PCR assays measuring *E2*/*E6*. We now demonstrate the feasibility of using the HPV MLPA assay for the analysis of FFPE material. Tables 1 and 2 summarize the results of MLPA analysis compared with the APOT, DIPS, and FISH data. These assays have been validated by comparison with other tests and are regarded as standards for the detection of viral integration.

3.1. Multiplex ligation-dependent probe amplification HPV typing and viral load

In the 13 FFPE samples with matching fresh frozen tissue, the MLPA assay identified 7 cases as HPV18 positive and 6 cases as HPV16 positive (see Table 1). No double infections with HPV16 and HPV18 were found. The matched fresh frozen tissue samples, of which some have been previously reported [24], showed HPV18 in 6 cases, HPV16 in 6 cases, and coinfection with HPV16 and HPV18 in 1 case. Typical examples of MLPA capillary electrophoresis peak profiles are shown in Fig. 1A and B, which illustrate the profiles for normal tissue and an HPV18-positive tumor (case 3). The biologic reproducibility of the MLPA assay to determine viral load was analyzed in tumor tissue fragments from 10 patients and is illustrated in Fig. 2A for the HPV16- and HPV18-positive patients. In 8 of these 10 patients, the variation of the viral load within the different tissue blocks from the same tumor was relatively low (within a factor of 2).

For series B, the samples were not microdissected, and therefore, the viral load may be influenced by contamination with stromal cells or HPV-negative normal epithelial cells. This is particularly obvious in cases 1, 3, 7, and 27 (see Table 2), which showed on average less than 1 HPV copy per genome. In series B, the HPV MLPA assay detected HPV16 in 14 cases, HPV18 in 4 cases, and double infection in 10 cases.

3.2. Comparison of the MLPA assay with APOT and DIPS methods to detect integration

Using the APOT assay, integration of the virus into the genome was found in all cases. In 4 cases, RNA transcripts from episomal HPV copies also were detected. A deletion of *E2* relative to *E6* was detected in all HPV18- and 5 of the 6 HPV16-positive cases with the MLPA assay. In 7 of these cases, episomal copies were also seen (mixed HPV). In 1 case, the *E2*/*E6* ratio indicated exclusively episomal viral copies. Fig. 3A to C shows examples of MLPA peak profiles of tumors with episomal and integrated viral copies. To identify the viral disruption sites, DIPS analysis was used to amplify the downstream fusion region between viral and cellular DNA. Sequence analysis of the DIPS amplification products showed the 3′ disruption site in the HPV genome to be different in all samples, ranging from position 1200 in the

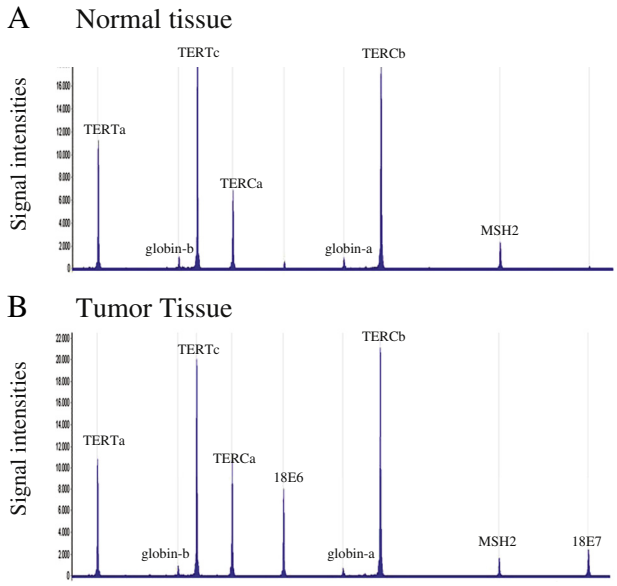

Fig. 1 Examples of MLPA capillary electrophoresis peak profiles from DNA isolated from normal (A) and tumor tissue (B).

HPV *E1* gene to approximately 4000 in the HPV *E5* gene (see Table 1). The difference in the *E2.1*/*E6* and *E2.2*/*E6* ratio measured with the MLPA assay was low in the various tumor biopsies from the same patients (see Fig. 2B and C). In case 6, a significant difference in the *E2.1*/*E6* and *E2.2*/*E6* ratios was found in the 2 biopsies. This resulted in the classification of 1 sample as integrated HPV and the other as both episomal and integrated HPV (mixed). In case 2, a difference in viral load for 1 of the 3 biopsies was measured, but not for the *E2.1*/*E6* and *E2.2*/*E6* ratios.

3.3. Comparison of the MLPA assay with FISH method to detect integration

Both series were analyzed by FISH. In FFPE sections, HPV integration manifests as punctate nuclear signals (see Fig. 3E). An example of episomal copies is depicted in Fig. 3D (including replication of the virus). In series A, viral integration was detected in 8 of 13 cases. In 5 cases, the FISH pattern was inconclusive for identifying viral integration or episomal copies, as no clear FISH pattern could be recognized. In all cases with an inconclusive pattern, we detected a minute viral load, ranging from 0.6 to 1.1 copies per genome. Because of the small number of FFPE tissue sections available, we could not repeat the hybridization experiments to improve immunochemical detection sensitivity. In this series, MLPA confirmed APOT and DIPS as well as FISH classification in 9 cases. Discrepancies between the assays were found in 4 cases, one of which showed concordance between the DIPS and the MLPA assay, with both unable to detect any viral integration. The other cases showed viral integration by all 3 of the reference methods but not by MLPA.

Table 2 summarizes the HPV-FISH and MLPA analysis of series B; in these cases, no APOT and DIPS data were available. The FISH method identified viral integration in 22 cases: the MLPA confirmed integration in 8 of these. In the 14 discordant cases, MLPA classified the samples as containing exclusively episomal viral copies. The 4 cases classified as episomal by FISH (no. 23-26) were also classified as episomal by MLPA. Three cases were classified as inconclusive by FISH: the MLPA detected a low viral load in these samples.

3.4. Ability of the MLPA assay to detect gain of TERC and TERT in FFPE material

The first MLPA experiments on FFPE material showed an altered peak profile for the human DNA targets compared with the profiles seen in the fresh frozen clinical samples. The capillary electrophoresis peak profiles for DNA isolated

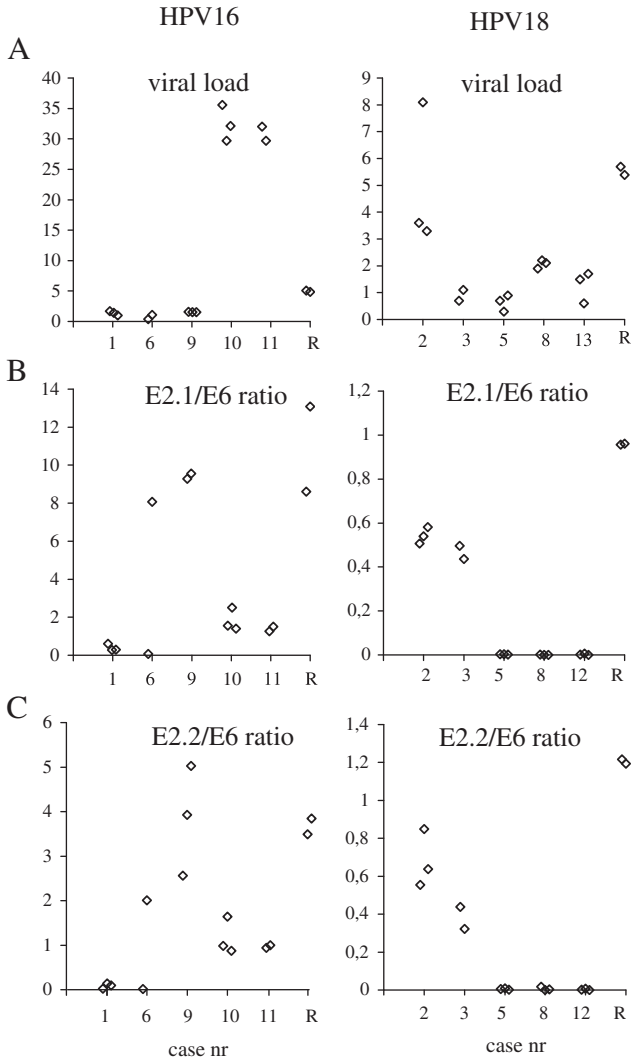

Fig. 2 A summary of the biological reproducibility of the measurement of viral load (A), *E2.1*/*E6* ratio (B), and *E2.2*/*E6* ratio (C) in multiple samples from the case series A. Abbreviations: case nr, case number; R, reference sample.

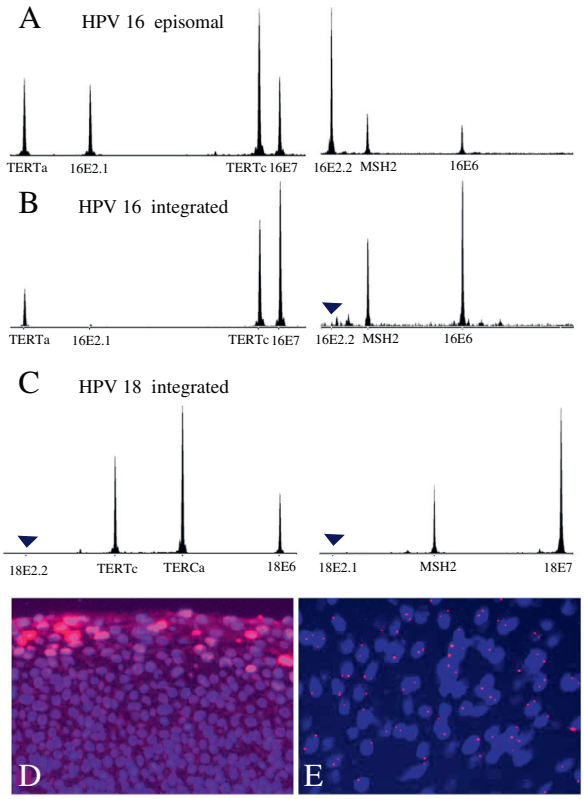

Fig. 3 A-C, Examples of MLPA capillary electrophoresis peak profiles for episomal (A, case 9), integrated HPV16 (B, case 1), and integrated HPV18 (C, case 3) in FFPE carcinomas from sample series A. Note the arrowheads indicating the absence of detectable *E2* signals in B and C. D and E, Examples of FISH patterns seen for episomal (D) and integrated HPV (E), case 25 from series B and case 10 from series A, respectively.

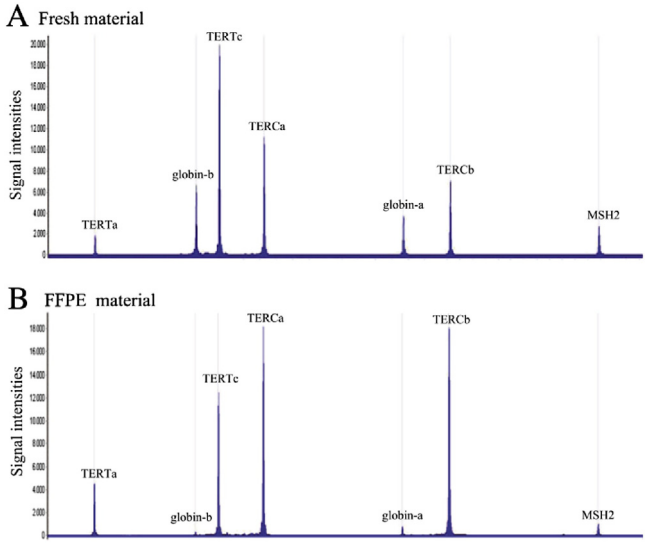

Fig. 4 Examples of MLPA capillary electrophoresis peak profiles for DNA isolated from normal fresh frozen (A) and FFPE (B) material.

from fresh and FFPE material are illustrated in Fig. 4A and B. The low intensity of *β-globin-a*, *β-globin-b*, and *MutS homolog 2* compared with the *TERT* and *TERC* peaks is clear. As a result, the signal intensity ratios for the *TERT* and *TERC* genes are different for these samples. Seven FFPE normal cervical samples showed average signal intensity ratios ± SD of 3.20 ± 1.85, 10.67 ± 4.04, 33.23 ± 16.23, and 26.50 ± 12.26 for *TERTa*, *TERTc*, *TERCa*, and *TERCb*, respectively, whereas the 7 previously described fresh frozen normal cervical samples showed average signal intensity ratios of 0.60 ± 0.18, 3.40 ± 1.14, 2.39 ± 0.67, and 1.53 ± 0.42 for *TERTa*, *TERTc*, *TERCa*, and *TERCb*, respectively [19]. These paraffin-embedded normal cervical samples were used to determine the reference threshold of value for the measurement of gain for *TERT* and *TERC*. Table 3 summarizes the *TERC* gains as detected by FISH and MLPA: 3 to 5 measurements were needed to measure the peak ratio between *TERCa/b* and the human references accurately. In these samples, the MLPA confirmed gain or absence of gain of *TERC* as detected by FISH in 12 of the 13 cases. In 1 case, the MLPA detected a gain, whereas FISH showed no gain. The utility of the MLPA (in its present form) to detect gain of *TERC* and *TERT* routinely is limited by reproducibility issues.

4. Discussion

This study demonstrates that the HPV MLPA assay can be applied to FFPE material for the detection of viral load and viral integration, whereas detection of gain of telomerase-related genes requires further technical improvements. Application of this assay to routinely processed material is a

Table 3 Gain of TERC by FISH and MLPA in selected samples from series B

Case	FISH TERC gain	MLPA TERC gain	Histologic classification
1	−	−	AdCa
2	−	−	SCC
7	−	−	AdCa
8	−	−	CIN3
10	−	−	SCC
11	−	+	SCC
15	−	−	SCC
16	+	+	SCC
17	+	+	SCC
18	−	−	CIN3
20	−	−	SCC
22	+	+	SCC
28	−	−	SCC

Abbreviations: AdCa, adenocarcinoma; CIN3, cervical intraepithelial neoplasia grade 3; FISH, fluorescence in situ hybridization; MLPA, multiplex ligation-dependent probe amplification; SCC, squamous-cell carcinoma; TERC, telomerase RNA component.

prerequisite for studies of the biology of HPV and for the diagnosis of these features of cervical lesions.

The main issue in the detection of viral integration is the variability in the outcome of the procedures used to assess the physical status. As described by Pett and Coleman [30], there are 2 categories of methods to detect viral integration, each with its own benefits and flaws. On the one hand, some protocols, including the DIPS assay, quantative polymerase chain reaction (qPCR), MLPA, and DNA FISH, detect early viral integration. Thus, these techniques will detect viral integration even in low-grade lesions. On the other hand, procedures that rely on the detection of transcriptionally active integrated virus, such as the APOT assay and RNA FISH, detect virus incorporated into the host genome at an advanced stage of carcinogenesis. Each of the assays has its own limitations. The assays with a high detection rate (APOT and DIPS) cannot be applied to FFPE tissue, limiting their use in daily practice. Although FISH can be applied to both fresh frozen and FFPE tissue, its interpretation is subjective. The HPV MLPA assay (and other qPCR assays) can be applied readily to fresh frozen tissue, cytologic samples, and FFPE material. However, it might not detect viral integration in the presence of predominantly episomal copies, as these will increase the *E2*/*E6* ratio. Because of these problems, several methods were used in this study to detect viral integration, as no single assay can be considered the criterion standard.

In series A, for which there was matched frozen tissue, the analysis of the physical status of the virus using the MLPA assay correlated well with the physical status, as determined with the APOT assay. Samples 6, 7, and 8 in series A showed integration by APOT/DIPS but a mixed pattern by MLPA. The viral disruption in those samples is at the end of *E2* (see Table 1). Integration was missed in only 1 case: a possible explanation for this is that the deletion is outside the *E2* MLPA target sequences. In this series, all the techniques, that is, APOT, FISH, and MLPA, detected viral integration efficiently.

In sample series B, for which only FFPE tissue was available, the FISH patterns suggested viral integration in 14 cases, whereas the MLPA suggested that only episomal copies were present. In 4 cases, FISH identified only integrated HPV, whereas the MLPA assay suggested that only episomal HPV was present. Similar to the discrepancy between the MLPA and APOT assays, this may be the result of a deletion outside the *E2* target sequences or retention of *E2* perhaps as part of integrated tandem HPV DNA repeats as is found, for example, in CaSki cells [31]. This was confirmed in a previous study, in which the MLPA could not detect viral integration in CaSki cells [19]. In several samples, FISH showed a mixed pattern, indicating the presence of both episomal and integrated HPV, but the integrated virus was not classified or recognized by the MLPA assay. The lack of detection of integrated HPV copies in samples where both integrated and episomal copies are present can be explained by the cutoff value applied to classify viral integration. In samples where less than 30% of the virus is integrated, this integration will not be detected because of technical issues, as described in the "Materials and methods" section. Therefore, these samples were classified as (predominately) episomal. However, the presence of integrated HPV, as detected by the MLPA assay, was confirmed by FISH in nearly all cases. In most of these cases, the MLPA assay also detected the presence of episomal HPV, whereas FISH detected exclusively integrated HPV. Possible explanations for the apparent absence of episomal copies by FISH analysis include the presence of a small proportion of episomal copies relative to integrated copies or the loss of episomal viral copies during FISH pretreatment [26]. Furthermore, it is important to recognize that the FISH pattern suggests viral integration but does not describe the fraction of cells that contain integrated virus.

Comparison of the capillary electrophoresis peak profiles from the normal samples of both FFPE and fresh samples shows that there are some appreciable differences in signal intensity. The FFPE normal cervix DNA shows a strong reduction in the *β-globin* signals and an increase in *TERC* signals compared with the fresh frozen normal lymphocyte DNA. These differences may be attributable to the use of different tissues but are most likely the result of the formalin fixation and paraffin embedding used to prepare the cervical samples.

The assessment of the different parameters in this assay may be influenced by these changes in the capillary electrophoresis peak profile, as these parameters are determined based on signal intensity ratios. The assessment of viral type and integration is not influenced by these changes because HPV signal intensities alone are used for this purpose, and these do not show any appreciable differences. For viral load, the signal intensity ratio between *E6* or *E7* and all human targets is used, and therefore, this change in capillary electrophoresis peak profile may have an effect. On the other hand, the reduction in *β-globin* signal intensity is counteracted by the increase in *TERT* and *TERC* signal intensity resulting in minor to no changes in signal intensity ratio for the assessment of viral load.

In conclusion, the HPV MLPA assay can be performed using routinely processed cervical lesions for the detection of viral load and HPV integration in a single assay. The ability to perform multiparametric analysis on routinely obtained clinical material will allow investigation of the role of these molecular parameters in HPV-associated carcinogenesis and further evaluation of their potential diagnostic role.

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