ORIGINAL ARTICLE

Use of multiple displacement amplification in the investigation of human papillomavirus physical status

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Background and Aims: The investigation of human papillomavirus (HPV) physical status in pre-invasive cervical lesions has been restricted by the small amounts of tissue available for study. Multiple displacement amplification (MDA), a phi29 DNA polymerase based whole genome amplification technique, has the potential to help resolve this problem by yielding large amounts of high molecular weight DNA from tiny starting quantities.

Methods: Firstly, a comparison was made of restriction endonuclease fragment patterns of DNA from seven different HPV types and corresponding MDA products. Secondly, *E6/E7* and LCR sequencing data from HPV16 recombinant plasmid and MDA copy DNA were correlated. Thirdly, DNA and MDA products from cervical cell lines (CaSki, HeLa, and SiHa that contain integrated HPV) and an invasive cervical carcinoma were analysed by Southern blot hybridisation. Fourthly, MDA product from CaSki cell DNA mixed with HPV18-plasmid DNA was tested for the demonstration of both episomal and integrated HPV. Finally, MDA products from HPV16 positive abnormal cervical cytological samples were assayed for integration by Southern blot hybridisation.

Results: DNA templates and MDA products yielded analogous data. Episomal and integrated HPV DNA were successfully detected by Southern blot assay of the cell line/HPV-plasmid model, and in MDA products of clinical samples.

Conclusions: These data show that MDA has considerable potential to assist in the investigation of HPV physical status; abundant (>40 µg) DNA can be generated with high fidelity from minuscule (50 ng) starting quantities, and both episomal and integrated HPV DNA are distinguishable in MDA products from solid tumours and cytological materials.

Human papillomavirus (HPV) integration into the host cell genome may be critical for progression of a cervical lesion to invasive cervical carcinoma (ICC).¹ However, the prevalence of HPV integration among cervical lesions has proven surprisingly difficult to estimate. HPV physical status has been investigated by Southern blot hybridisation,^{2–4} PCR assay of *E2* region integrity,^{5–6} quantitative PCR assay of the ratio of *E2* to *E6/E7* region amplicons,^{7–8} and by the amplification of papillomavirus oncogene transcripts (APOT) technique, where integration is measured by the presence of cellular sequence transcripts contiguous with HPV *E6/E7* mRNA.⁹ Estimates of HPV integration in cervical disease vary according to the method employed and are especially dissimilar with respect to cervical intraepithelial neoplasia (CIN); estimates range from 10% to 100% in CIN III, and from 65% to 100% in ICC.¹⁰ One factor limiting the study of HPV integration in CIN lesions is the small amounts of fresh tissues (and therefore high molecular weight DNA) available for experimental analysis.

Multiple displacement amplification (MDA) is an isothermal whole genome amplification technique that utilises phi29 DNA polymerase. Phi29 DNA polymerase has high processivity (supporting amplification of templates up to 100 kb), high proofreading capacity, and strand displacement activity.^{11–12} Additionally, the MDA technique results in uniform amplification across the entire genome with minimal locus bias.¹³ MDA has been validated as a technique for use in genomic studies,¹⁴ especially for single nucleotide polymorphism analyses,¹⁵ and has been applied to a wide range of biological samples including a single bacterium,¹⁶ and single sperm.¹⁷

In this study, we have examined the MDA technique as an aid for investigating HPV integration. Firstly, restriction

endonuclease fragment patterns from recombinant HPV plasmid DNA samples and MDA products were compared to determine the effectiveness of MDA for synthesising circular double-stranded DNA (i.e. episomal HPV). Secondly, to better examine the fidelity of MDA, HPV16 *E6/E7* region and long control region (LCR) sequences were PCR amplified from HPV16 recombinant plasmid and MDA copy, and the amplicons were compared following cycle-sequencing. Thirdly, DNA extracts and MDA products from an ICC and cervical cell lines containing integrated HPV were evaluated by Southern blot analyses. Fourthly, MDA product from a mixture of HPV18 plasmid DNA and CaSki cell DNA was tested to confirm amplification of both integrated and episomal HPV. A novel potential source of pre-invasive tissues for HPV integration assay might be cells left over following routine Papanicolaou (Pap) testing; thus, fifthly, cervical cytological sample MDA products were assayed by Southern blot hybridisation.

MATERIALS AND METHODS

DNA, cell line, and clinical specimens

HPV recombinant plasmids

HPV types 16 and 45 were gifts from Dr E-M de Villiers, Deutsches Krebsforschungszentrum, Heidelberg, Germany. HPV33 was received courtesy of Dr Gerard Orth, Institut Pasteur, Paris, France. HPV types 6, 18, 31, and 43 were

Abbreviations: APOT, amplification of papillomavirus oncogene transcripts; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; ICC, invasive cervical carcinoma; LCR, long control region; LEEP, loop electrosurgical excision procedure; MDA, multiple displacement amplification; Pap, Papanicolaou

Table 1 Comparisons of HPV16 base sequences from recombinant plasmid template DNA, MDA copy, and published sequences

Bases compared	HPV16	MDA vs template		Template vs published		MDA vs published	
		N	B	N	B	N	B
n=814	E6/E7	1	1	1	1	2	0
n=970	LCR	0	3	4	2	4	1

HPV, human papillomavirus; MDA, multiple displacement amplification; LCR, long control region. HPV16 E6/E7 and LCR regions were PCR amplified from HPV16 template DNA and the MDA copy for sequencing analyses. Differences were recorded as B (base non-matches) and N (raw sequence data output recorded as N rather than A, C, G, or T) changes.

obtained from the American Type Culture Collection (ATCC), Manassas, VA.

Cell lines

CaSki cells that contain ~600 copies of HPV16 integrated at 12 or more sites, HeLa cells that possess ~50 copies of HPV18 at five locations, and SiHa cells that contain one copy of the HPV16 genome integrated per chromosome 13q21–31,¹⁸ were obtained from the ATCC.

Clinical samples

One HPV18 positive ICC and 20 HPV16 positive abnormal cervical cytological samples were analysed with approval from the University of Vermont Institutional Review Board.

Multiple displacement amplification

MDA was performed using a REPLI-g kit (Qiagen Inc., Valencia, CA, USA). Template DNA (HPV recombinant plasmid, cell line, or clinical sample DNA) 50–100 ng was alkaline denatured and incubated for 16 h with phi29 DNA polymerase

reagents; reactions were terminated by incubation at 65°C for 3 min. Dual amplification of integrated and episomal HPV was modelled by MDA of 100 ng CaSki cell DNA mixed with 1 ng of HPV18 recombinant plasmid (pBR322) DNA.

Restriction endonuclease digests

HPV recombinant plasmids and MDA counterparts (500 ng) were digested with *Bam*HI, *Eco*RI, *Hae*III, *Hind*III, or *Pst*I (New England BioLabs, Ipswich, MA, USA). Cell line DNA/MDA samples (10 µg) were treated with *Bam*HI, *Hpa*II, *Kpn*I, *Msp*I, *Pst*I, and *Xho*II. HPV16 positive cervical cytological MDA products (10 µg) were digested with *Bam*HI, *Hind*III, *Kpn*I, and *Stu*I. The HPV18 positive ICC samples (10 µg) were digested with *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Nci*I, and *Xba*I. MDA products (10 µg) from the CaSki/HPV18 DNA combination were digested with *Bam*HI, *Hind*III, and *Kpn*I. Digests were performed overnight (16 h) at 37°C using 5–10 U enzyme per 1 µg DNA.

Southern blot transfer and hybridisation

All DNA/MDA digest samples were purified by column treatment (DNA Clean and Concentrator-25; Zymo Research, Orange, CA, USA) prior to gel electrophoresis. DNA was transferred to nylon membrane (BioBond-Plus; Sigma-Aldrich, St Louis, MI, USA) by 1-hour electrotransfer using a Genie Blotter apparatus (Idea Scientific Company, Minneapolis, MN, USA), and denatured by rinsing the membrane for 10 min in 0.4M sodium hydroxide, followed by a 10-min neutralising rinse with 2×SSC pH 7.0. Membranes were transferred directly into PerfectHyb Plus hybridisation buffer (Sigma-Aldrich) and pre-hybridised for 15 min at 68°C in a hybridisation oven (Robbins Scientific-SciGene, Sunnyvale, CA, USA). Blots were hybridised for 16 h at 68°C following the addition of full-length HPV probe labelled using the α-³²P dCTP Redivue/Rediprime II Random Prime Labelling System (Amersham Biosciences, Piscataway, NJ, USA). Blots were washed twice for 20 min under high-stringency conditions (0.5×SSC, 0.1% SDS at 68°C). Hybridisation signals were detected by exposing blots to Kodak BioMax MS film with Kodak BioMax Transcreen HE (Kodak, New Haven, CT, USA) intensifying screens at –80°C for 12–72 h.

PCR of HPV-16 sequences

Primers were designed to amplify: (i) an 860 base pair sequence of the E6/E7 region (forward: 5'-TTG AAC CGA AAC CGG TTA GT-3'; reverse: 5'-CTC TTC CCC ATT GGT ACC TG-3'); and (ii) a 1034 base pair sequence of the LCR (forward: 5'-CAG TTT CCT TTA GGA CGC AAA-3'; reverse: 5'-TAA CTT TCT GGG TCG CTC CT-3'). A 50 µl PCR consisted of 25 ng of HPV16 recombinant DNA or 25 ng of MDA copy in HotStarTaq Buffer (Qiagen Inc.), 1.0 µM each primer, 4 mM MgCl₂, 200 µM dNTPs, and 1 U HotStarTaq DNA polymerase (Qiagen Inc). Cycling coordinates comprised 15 min at 95°C (to activate the HotStarTaq DNA

Figure 1 HPV (Human papillomavirus) recombinant plasmid restriction endonuclease digestion patterns. HPV (types 6, 16, 18, 31, 33, 43 [genome fragments A and B cloned in separate plasmid vectors], and 45) recombinant plasmids digested with *Hae*III, which cuts HPV at multiple sites. Identical fragment patterns were recorded for multiple displacement amplification product (M) and template recombinant plasmid (P). L, molecular weight ladder (1 Kb Plus DNA Ladder; Invitrogen, Carlsbad, CA, USA).

Figure 2 SiHa and CaSki (HPV16), and HeLa (HPV18) cell line DNA after restriction digest with *Pst*I and *Xho*II and Southern blot assay. Identical fragment patterns (detailed in kilobase pairs) were recorded for genomic cell DNA template (G) and multiple displacement amplification product (M).

polymerase) and 35 cycles consisting of 1 min at 94°C, 1 min at 55°C, and, 2 min at 72°C.

Sequencing

E6/E7 and LCR PCR products were excised from an agarose gel and purified using a Qiaex II gel extraction kit (Qiagen Inc.). Cycle sequencing analyses (Big Dye v3.1 chemistry and 3130 xI Genetic Analyzer technology (Applied Biosystems, Foster City, CA, USA)) were performed by the Vermont Cancer Center DNA Analysis Core Facility laboratory.

RESULTS

HPV recombinant plasmid DNA and MDA restriction fragment patterns

Identical restriction fragment patterns were observed for each HPV type/plasmid combination tested after digestion with *Bam*HI, *Eco*RI, *Hae*III, *Hind*III, or *Pst*I. Figure 1 details data

Figure 3 Genomic (G) and MDA (M) SiHa and CaSki cell DNA Southern blot hybridisation fragment patterns (detailed in kilobase pairs) after treatments with isochizomers *Hpa*II (methylation sensitive) and *Msp*I (methylation insensitive) showing the loss of epigenetic information following MDA (multiple displacement amplification).

obtained after *Hae*III treatment. *Hae*III restricts HPV genomes at multiple sites.

Comparison of *E6/E7* and LCR sequences

Sequence data were obtained for 814 *E6/E7* and 970 LCR individual bases. Dissimilarities (MDA copy versus template) were recorded in terms of bases in the sequencing data output registered as “N” (e.g. N in the MDA sequence and A, C, G, or T in the HPV16 plasmid data), or in terms of base “B” non-matches (e.g. C in the MDA copy but A, G, or T in the template). Three assessments were made (table 1). Firstly, the MDA PCR product data were compared directly to the template HPV16 recombinant plasmid sequences. The MDA *E6/E7* data differed from the template HPV16 data by one “N” record, and by one “B” non-match. There were three “B” changes comparing the LCR MDA data with the LCR template and no “N” differences. Secondly, the data from the template HPV16 sequences were matched to the published ones.¹⁹ The *E6/E7* data were at variance with the published sequences by one “N” record and one “B” non-match. The LCR template data deviated from the published data by four “N” records and two “B” changes. Thirdly, the data from the HPV16 MDA products were compared to the published sequences. The *E6/E7* data differed from the published sequences by two “N” records but there were no “B” non-matches. The LCR data deviated from the published data by four “N” records and one “B” change.

Southern blot hybridisation assay of CaSki, HeLa, and SiHa, and CaSki/HPV18 plasmid samples

Cell line DNA and MDA products yielded corresponding blot hybridisation patterns after *Bam*HI, *Hpa*II, *Kpn*I, *Msp*I, *Pst*I, or *Xho*II treatment (figs 2 and 3). Dissimilar patterns were noted with *Hpa*II (fig 3). Both *Hpa*II and *Msp*I recognise the sequence CCGG; however, *Hpa*II is methylation sensitive. Accordingly, identical fragments were detected in the MDA samples after *Hpa*II or *Msp*I treatments and in the cell sample after *Msp*I, whereas the cell line DNA showed higher molecular weight bands after *Hpa*II treatment.

The CaSki/HPV18-plasmid MDA blot was first hybridised using an HPV18 probe; after probe stripping, membranes were re-hybridised using an HPV16 probe. This approach facilitated the appreciation of the detection of integrated and episomal HPV simultaneously amplified by MDA (fig 4).

Figure 4 Southern blot of multiple displacement amplification (MDA) products obtained from a mixture of 100 ng of Caski cell DNA and 1 ng of HPV18-pBR322 DNA. Samples (10 µg) were digested with *Bam*HI (B), *Hind*III (H), and *Kpn*I (K). The blot was first hybridised with an HPV18 probe (18), and after probe stripping, with an HPV16 probe (16). The data show that episomal and integrated human papillomavirus (HPV) can be simultaneously amplified by the MDA technique. Fragment sizes are detailed in kilobase pairs.

Southern blot hybridisation assay of clinical samples

Matching restriction hybridisation patterns were obtained for DNA extracted directly from an HPV18 positive ICC and for the corresponding MDA product (fig 5). The data are consistent with the presence of integrated HPV; a single band of 7857 base pairs following *Eco*RI, *Eco*RV, or *Nci*I treatments that each linearise the (episomal) HPV18 genome was not observed. Similarly, predicted "episomal" two-fragment patterns (6810 and 1047 base pairs) following *Bam*HI digestion, and three-band patterns (3998, 2448, and 1411 base pairs) following *Xba*I digestion, were not detected. The high molecular weight DNA observed after treatment with *Hind*III, an HPV18 non-cutter, is also consistent with the presence of integrated HPV.

MDA products from 20 HPV16 positive cervical cytological samples were also assessed by blot hybridisation. The amount of DNA extracted from the cytological samples was insufficient for a direct comparison with the MDA product. Nineteen MDA samples yielded the expected fragment sizes for episomal HPV following *Bam*HI, *Kpn*I, and *Stu*I treatments (fig 6A); the higher molecular weight DNA observed after treatment with *Hind*III, which does not restrict HPV16, is interpretable as evidence that the MDA technique also reproduces HPV concatamers that can confuse the interpretation of HPV physical status unless multiple Southern blot restriction hybridisation assays are performed.³ One sample (ASC-H cytology with follow-up CIN

III histology) showed a pattern indicating the presence of episomal and integrated HPV (fig 6B); these data were reproducible on repeat MDA and restriction hybridisation assay of the cytological DNA extract.

DISCUSSION

This study has validated the MDA technique as an aid for HPV integration analysis. Tests comparing HPV recombinant plasmid DNA, cell line genomic DNA, and ICC DNA with the corresponding MDA copies consistently showed analogous data. Although non-similar data were obtained using a methylation sensitive restriction endonuclease (*Hpa*II, fig 3), this is simply because MDA is a DNA sequence specific technique; possibly MDA might have utility in tumour methylation studies by allowing the synthesis of non-methylated whole genome sequences for (baseline) comparison with normal and tumour methylation patterns. The one base non-match recorded between 814 *E6/E7* HPV16/plasmid and the MDA DNA sequences, and the three bases non-matched comparing 970 LCR sequences (table 1), might be accountable by methodology; the sequences analysed were PCR products and possibly the *Taq* DNA polymerase enzyme introduced sequence errors. The fact that the *E6/E7* and LCR sequences from the MDA product better matched the published sequences than the PCR product obtained directly from the HPV16 recombinant plasmid is consistent with this possibility (table 1). Nevertheless, the simplicity of the MDA technique and the abundant MDA product yield (40–50 µg DNA per REPLI-g reaction) allow numerous assays and repeat tests for confirmation of HPV physical status (episomal, episomal plus integrated, or integrated) and exclusion of the possibility that a "mutant" MDA sequence resulted in aberrant restriction fragment patterns.

Of particular note in this study is the combination of MDA with DNA extracts from cervical cytological cell samples left over after routine Pap smear testing. This allowed for the first time (indirect) Southern blot hybridisation analysis of cytological specimens and showed that HPV integration can be detected by this approach. The potential use of cytological samples in the study of HPV integration is important given the limited availability of pre-invasive tissues for experimental assay. The trend towards conservative biopsy and loop

Figure 5 Southern blot hybridisation data from an HPV18 positive invasive cervical carcinoma (tumour vs multiple displacement amplification DNA copy) showing integrated human papillomavirus (HPV) sequences. Analogous restriction fragment patterns (detailed in kilobase pairs) following digestion with *Hind*III (H), which is an HPV18 non-cutter; *Nci*I (N), *Eco*RI (E1), and *Eco*RV (E5), which each digest the HPV18 genome once; *Bam*HI, which has two restriction sites; and *Xba*I, which has three restriction sites.

Figure 6 Episomal (A) and episomal and integrated (B) HPV16 demonstrated after Southern blot assay of multiple displacement amplification products from abnormal cervical cytological sample DNA extracts (fragment sizes detailed in kilobase pairs). H, *Hind*III (non-cutter); B, *Bam*HI, S, *Stu*I (one restriction site); K, *Kpn*I (two restriction sites).

Take-home messages

- Human papillomavirus integration can be investigated in solid tumours or cervical cytology specimens by Southern blot hybridisation analysis of sample DNA multiple displacement amplification (MDA) products.
- MDA allows high fidelity, high yield whole genome amplification, supporting its use in any pathology research where tissue/DNA samples are limiting.

electrosurgical excision procedures (LEEP), together with the clinical requirement to review all biopsy/LEEP tissues, limits the collection of fresh specimens for research. Additional MDA/HPV integration studies of a larger set of cytological samples are in progress in our laboratory.

In conclusion, the abundant high molecular weight high fidelity DNA procurable by the MDA technique supports the use of MDA in the investigation of HPV integration, and, by extension, MDA has the potential for application in any pathology research where fresh tissue/DNA availability is limiting.

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