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Archival cervical smears: a versatile resource for molecular investigations

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Archival cervical smears: a versatile resource for molecular investigations

Archival cervical smears represent a huge resource of pathological specimens. This, together with long clinical follow-up data, makes archival smears the most valuable resource for cervical cancer research. Despite this huge potential, only a few molecular investigations have been carried out based on archival smears. It has been shown that archival smears can be used for amplification of genomic sequences by polymerase chain reaction (PCR). However, it is unknown whether PCR can be applied to minute dyskaryotic cells microdissected from archival cervical smears and whether these archival materials are suitable for reverse transcription PCR (RT-PCR). To address these issues, we prepared DNA and RNA samples from dyskaryotic cells microdissected from archival cervical smears with a storage time of 11 years and systematically tested the extent that these materials can be used for PCR-based molecular investigations at both DNA and RNA levels. Our results showed that a crude DNA preparation simply by proteinase K digestion was suitable for PCR amplification of genomic sequences. By targeting the amplified genomic sequence to 250 bp or less, most if not all archival smears could be used for PCR and are therefore suitable for screening gene mutations and loss of heterozygosity, human papillomavirus typing, etc. Purified DNA samples from microdissected dyskaryotic cells were adequate for restriction enzyme digestion and could be used for a PCR-based clonality analysis of the androgen receptor gene. Finally, RNA samples extracted from dyskaryotic cells microdissected from archival smears were adequate for RT-PCR as long as a gene-specific primer was used for the RT reaction and the target sequence was restricted to 150 bp or less. In summary, our results demonstrated that archival cervical smears are suitable for a range of molecular investigations at both DNA and RNA levels. The potential gain of knowledge on cervical cancer by the molecular study of archival smears is immense.

Keywords: archival cervical smear, PCR, RT-PCR

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INTRODUCTION

Carcinoma of the uterine cervix is one of the most common malignancies among women worldwide and its mortality rate is high.[1] Most cervical carcinomas are believed to derive from preneoplastic epithelial lesions known as cervical intraepithelial neoplasia (CIN).[2] To identify these preneoplastic lesions, the Papanicolaou (Pap) smear test was introduced in 1943 and has been subsequently used as a screening method. Nationwide screening programmes for cervical cancer were set up in 1988 in the United Kingdom and have permitted a dramatic reduction in the cervical cancer mortality rate of up to 70%.[1]

Cervical smears represent one of the largest resources of pathological specimens. With appropriate long-term clinical follow-up, the archival cervical smears could be the most important specimens for cervical cancer research including molecular investigations of genetic and epigenetic factors. Despite this huge potential, only a few molecular investigations have been carried out based on archival cervical smears. Several studies have shown that archival cervical smears can be used for amplification of genomic sequences by polymerase chain reaction (PCR).[3–7] These studies were based on a relatively large population of mixed normal and abnormal cells collected from cervical smears. It is unknown whether PCR can be applied to minute dyskaryotic cells microdissected from archival smears because these cells are the main research interest. In addition, it remains to be tested whether archival cervical smears are suitable for PCR-based RNA analysis. To address these issues, we prepared DNA and RNA samples from dyskaryotic cells microdissected from archival cervical smears and systematically tested to what extent such archival materials can be used for PCR-based molecular investigations.

MATERIALS AND METHODS

Materials

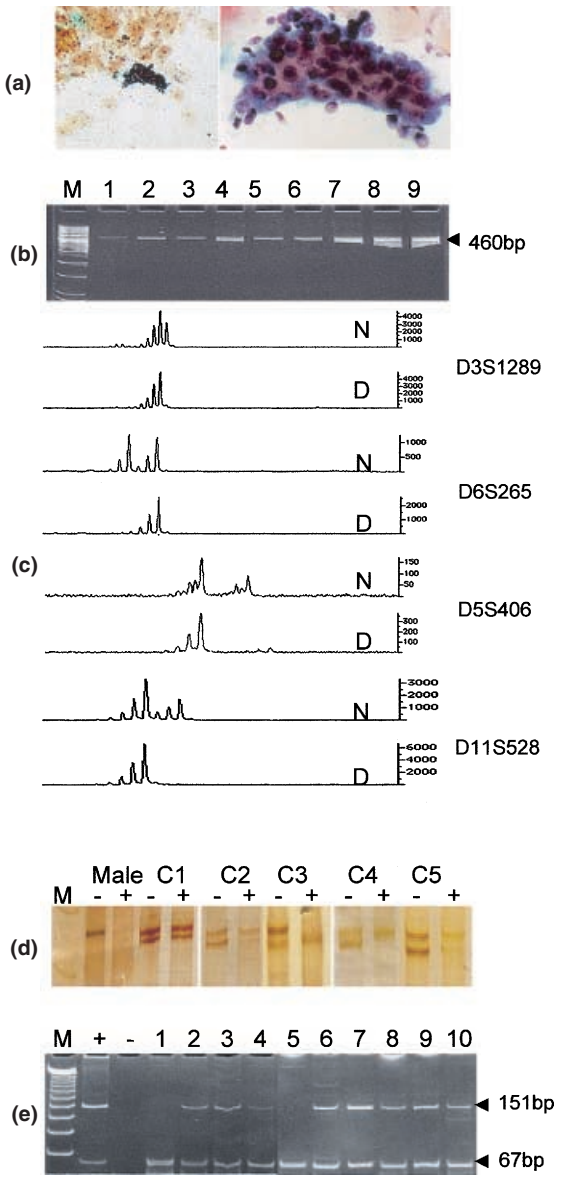
Cervical smears with a storage time of up to 11 years from 30 patients (seven mild dyskaryosis, 11 moderate dyskaryosis and 12 severe dyskaryosis) were retrieved from the cytopathological files of the Department of Histopathology, University College London.

Figure 1. (a) Microdissection of dyskaryotic cells from archival smears. An example of clustered dyskaryotic cells microdissected from routinely prepared cervical smear glass slides; (b) examples of polymerase chain reaction (PCR) amplification of BCL10 coding exon 3 (460 bp) from crude DNA preparations of microdissected dyskaryotic cells. M: DNA marker; 1–9: different cases. (c) Examples of loss of heterozygosity (LOH) analysis of four microsatellite loci from crude DNA preparations of microdissected cells. Dyskaryotic cells show an allelic deletion in comparison with normal cells. N: normal cells, D: dyskaryotic cells. (d) Clonality analysis of dyskaryotic cells from archival cervical smears. Purified DNA samples were prepared from microdissected dyskaryotic cells. Male DNA shows lack of amplification after *Hap*II digestion as expected, indicating complete digestion with the *Hap*II restriction enzyme. C1 is a cervical intraepithelial neoplasia (CIN)1 and shows a polyclonal pattern. C2 and C3 are moderate dyskaryosis , and C4 and C5 are severe dyskaryosis . They show a monoclonal pattern. M: DNA marker; –: without *Hpa*II digestion; + : with HpaII digestion; Male: male DNA; E: RT-PCR of the glucose-6-phosphate dehydrogenase (G6PD) gene of dyskaryotic cells microdissected from archival smears. All cases show amplification of a 67-bp product, and eight of 10 cases display amplification of 151-bp products. + : positive control, –: negative control.

The morphology of these cervical smears was reviewed. Formalin-fixed and paraffin-embedded tonsils from two male patients were also retrieved and used as controls.

Microdissection of dyskaryotic cells from cervical smears

Dyskaryotic cells were identified and marked with a diamond pen on the reverse side of the slide. The slide was incubated in xylene for 2–5 days to remove the coverslip, and then washed in 100% and 70% ethanol. Dyskaryotic cells (10^5–10^7) were microdissected using



glass transfer pipettes, as described previously (Figure 1a).[8] Where appropriate, normal epithelial cells were also dissected from the cervical smears.

Crude DNA preparation and polymerase chain reaction of genomic sequences

DNA extraction

Briefly, microdissected cells were digested with 100 μg/mL proteinase K in 100–200 μL of 1 × PCR buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 1.0% Triton X-100 at 56°C for 16–20 h in a volume of 50–100 μL depending on the number of cells. The digests were heated at 95°C for 10 min to inactivate proteinase K and centrifuged to remove cell debris.

Polymerase chain reaction of the p53 and BCL10 genes

Three μL of crude DNA preparations were used for PCR amplification of three genomic segments of variable sizes: p53 exon 8 (185 bp), p53 exon 5 (245 bp)[9] and BCL10 coding exon 3 (460 bp) (Table 1).[10] Polymerase chain reaction was performed in a thermal cycler (Hybaid, UK) using a 'hot-start touch-down' programme with an annealing temperature starting at 60°C, reducing 2°C every cycle until 55°C for 38 cycles. Polymerase chain reaction products were analysed by electrophoresis on 1.5% agarose gels.

Table 1. DNA oligos used for polymerase chain reaction

Gene target	Primer name	Primer sequence	Fragment amplified (bp)
p53	Exon 5 S	5′-TTCCTCTTCCTGCAGTACTC-3′	245
	Exon 5 AS	5′-ACCCTGGGCAACCAGCCCTGT-3	
	Exon 8 S	5′-CCTATCCTGAGTAGTGGTAA-3′	185
	Exon 8 AS	5′-GTCCTGCTTGCTTACCTCGC-3′	
BCL10	Exon 3 S	5′-TTAACAAGTCACAAGATGGACAGTG-3′	460
	Exon 3 AS	5′-CATTAAAAATTAAAAGGCAATAAAGTG-3′	
D3S1289	S*	5′-GCAACTTGTAAGAGAGCATTCA-3′	259
	AS	5′-ACATTACAGAAATGTGATGCA-3′	
D5S406	S*	5′-TGCCAATACTTCAAGAAAAACA-3′	188
	AS	5′-ACTTGGGATGCTAACTGCTG-3′	
D6S265	S*	5′-ATCACCCCCCTCACACAC-3′	100
	AS	5′-TCTAATCGAGGTAAACAGCAGA-3′	
D11S528	S*	5′-GCCTAACTAATGGTGTCCCC-3′	150
	AS	5′-GACCCCAGTGTGAGATGAAT-3′	
AR	S	5′-TCCAGAATCTGTTCCAGAGCGTGC-3′	220
	AS	5′-GCTTGGGGAGAACCATCCTCAC-3′	
G6PD	G6PD-S	5′-GGCAACAGATACAAGAACGTGAA-3′	
	G6PD-AS1	5′-CGCAGAAGACGTCCAGGAT-3′	67
	G6PD-AS2	5′-CCAGCTCAATCTGGTGCAG-3′	151
	G6PD-AS3	5′-CCCTCATACTGGAAACCCACT-3′	242

*The primer was labelled with florescent dyes: FAM, HEX, NED and FAM, respectively.
G6PD – glucose-6-phosphate dehydrogenase.

Loss of heterozygosity analysis

Four microsatellite loci of various chromosomes were randomly selected and subjected to PCR with fluorescently labelled primers (Table 1). Polymerase chain reaction was carried out in a thermal cycler using a 'hot-start touch-down' programme, as above. Polymerase chain reaction products were first confirmed on agarose gels and then analysed on an ABI Prism 377 DNA sequencer (Applied Biosystems Inc., San Francisco, CA, USA) with GeneScan software (version 3.0, Applied Biosystems Inc.). Dyskaryotic and normal cells from the same case were analysed in parallel.

Purified DNA and clonality analysis

DNA purification

For molecular analysis involving restriction enzyme digestion, such as clonality analysis of the X-chromosome inactivation pattern, a highly purified DNA sample is necessary. The microdissected cells were washed in 1 mL of 10 mM Tris (pH 8.0) and 1 mM of EDTA at 4°C for 1 h to remove the stain and then centrifuged at 13 000 g for 5 min. The cell pellets were digested with 100 μg/mL proteinase K in 50 μL of 1 × PCR buffer at 56°C for 60 min. Protein was precipitated with protein precipitation solution (Promega, Southampton, UK) and then removed by centrifugation at 25 000 g for 20 min at 4°C. DNA in the supernatant was precipitated with isopropanol, washed in 70% ethanol, air dried and dissolved in 20 μL of distilled water. DNA samples were similarly purified from two formalin-fixed and paraffin-embedded tonsils of male patients.

Restriction enzyme digestion

Four μL of purified DNA was digested with and without 12 units *Hpa*II in a total of 10 μL reaction mixture at 37°C for 16–20 h. The digestion was terminated by heating at 95°C for 10 min and the reaction mixtures were centrifuged. For each experiment, one male DNA sample was similarly digested as a control.

Polymerase chain reaction of the androgen receptor gene

For clonality analysis, 5 μL of *Hpa*II digested and mock-digested DNA samples were used for PCR of the highly polymorphic CAG repeat of the androgen receptor (AR) gene. The amplification was carried out in a 25-μL reaction mixture containing 1 × PCR buffer, 4 pmol of each primer (Table 1), 200 μM of each dNTP and 1.5 mM of $MgCl_2$. Polymerase chain reaction was performed in a thermal cycler using a 'hot-start touch-down' programme as above but with 35 cycles at an annealing temperature of 57°C. Polymerase chain reaction products were separated on 7% polyacrylamide gels by electrophoresis and visualized by sliver staining (Pharmacia-Amersham, Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK). The sample was judged as monoclonal if the intensity of one of the two alleles was reduced visually by more than 50%, whereas the sample was considered as polyclonal if both alleles displayed similar intensity.

Reverse transcription PCR of the glucose-6-phosphate dehydrogenase gene

RNA extraction

Total RNA extraction from microdissected dyskaryotic cells was carried out using an Ambion Kit (AMS Biotechnology, Oxon, UK). Briefly, cells were digested in 100 μL

of digestion buffer containing 100 μg of proteinase K at 45°C for 2 h. The digests were added with 600 μL of RNA extraction buffer and incubated at room temperature for 5 min followed by extraction with 700 μL of acid phenol : chloroform. The aqueous layer of the extracts was recovered into a fresh tube and 1 μL of linear acrylamide (5 μg/μL) was added as a carrier. The RNA was precipitated with an equal volume of isopropanol at −20°C for 2 h and pelleted in a microcentrifuge at 4°C for 15 min. The RNA pellet was then washed with 70% alcohol, air-dried and dissolved in 20 μL of RNA Storage Solution™ (Ambion (Europe) Ltd., Huntingdon, Cambridgeshire, UK).

Reverse transcription PCR

The extracted RNA was used for reverse transcription and subsequent cDNA amplification of the glucose-6-phosphate dehydrogenase (G6PD) gene. To eliminate amplification from contaminating genomic DNA, the sense primer was designed to span an exon–exon boundary. The sense primer was combined with three antisense primers, which should have yielded PCR products of 67 bp, 151 bp and 242 bp, respectively (Table 1).

One-step reverse transcription PCR (RT-PCR) was performed using a Qiagen OneStep RT-PCR Kit (Qiagen, West Sussex, UK). Briefly, G6PD primers were added to the RT-PCR mixture at a final concentration of 0.6 μM for the sense primer and 0.15 μM for each of the three antisense primers, and 5 μL of the above-extracted total RNA was used as a template for a 25-μL RT-PCR reaction. The thermal cycler was programmed to start with reverse transcription at 50°C for 30 min and then heating at 95°C for 15 min to inactivate Omniscript™ (QIAGEN Ltd., Crawley, West Sussex, UK) reverse transcriptase and activate HotStartTaq™ (QIAGEN Ltd.) DNA polymerase. This was followed by a 'touch-down' PCR programme: denaturation at 94°C for 30 s, annealing at 65–57°C (one degree down each cycle) for 30 s and extension at 72°C for 30 s, and then 30 cycles with annealing at 56°C. A final extension step at 72°C for 10 min concluded the reaction. Polymerase chain reaction products were analysed on 10% polyacrylamide gels and visualized by ethidium bromide staining.

RESULTS

Crude DNA preparations are suitable for PCR of genomic sequences

To test the suitability of crude DNA preparations from archival cervical smears for PCR-based molecular investigations, we first performed PCR amplification of three gene segments of various sizes. When gene fragments less than 250 bp were examined, each of the 21 cervical smears examined showed a product of expected size for both p53 exon 8 (185 bp) and exon 5 (245 bp) PCRs. When a significantly longer fragment, BCL10 coding exon 3 (460 bp), was tested, 17 of the 21 (81%) samples yielded a product of expected size (Figure 1b).

Similarly, PCR amplification of four microsatellites ranging from 100 to 260 bp was successful in all 20 cases examined. By analysis of dyskaryotic cells and normal epithelial cells of the same case in parallel, loss of heterozygosity (LOH) was detected (Figure 1c). Table 2 summarizes the heterozygous rate and frequencies of LOH of these loci.

Table 2. Summary of loss of heterozygosity of the microsatellites studied

	Mild dyskaryosis		Moderate dyskaryosis		Severe dyskaryosis	
Locus	Heterozygosity	LOH	Heterozygosity	LOH	Heterozygosity	LOH
D3S1289	4/4	1/4	8/8	6/8	8/8	5/8
D5S406	3/4	0/3	7/8	3/7	6/8	2/6
D6S265	3/4	0/3	6/8	0/6	7/8	0/7
D11S528	3/4	3/3	8/8	3/8	8/8	2/8

LOH – loss of heterozygosity.

Purified DNA is adequate for PCR-based clonality analysis

Polymerase chain reaction-based clonality analysis of the AR gene was carried out in 10 cases including three mild dyskaryosis, three moderate dyskaryosis and four severe dyskaryosis. In each experiment, *Hpa*II-digested male DNA samples, as expected, showed no PCR amplification of the AR gene, indicating complete digestion (Figure 1d). Dyskaryotic cells from the cervical smears showed monoclonal patterns in 4/4 severe dyskaryosis and 2/3 moderate dyskaryosis , but polyclonal patterns in the remaining moderate dyskaryosis and the three mild dyskaryosis samples (Figure 1d).

Reverse transcription-PCR of the G6PD gene

To examine whether archival cervical smears are suitable for PCR-based RNA detection, we performed RT-PCR of the G6PD gene in 10 cases of CIN3. Three segments of the gene ranging from 67 bp to 242 bp were amplified in a single tube in each case. All cases showed amplification of an expected 67-bp product, and eight of the 10 cases yielded an expected 151-bp product (Figure 1e). However, the 242 bp fragment was not amplified in any of the cases examined. To ascertain further that the failure of the amplification of the 242-bp product resulted from RNA degradation rather than failed amplification in a multiplex PCR reaction, a separate RT-PCR with a single pair of primers for the 242 bp fragment was performed. The single set PCR consistently showed a negative result despite amplification of an expected product from positive controls.

DISCUSSION

Previous studies have shown that PCR amplification of genomic DNA sequences could be achieved with DNA samples prepared from a relatively large population of mixed normal and abnormal cells collected from archival cervical smears.[3–7] Our present study further demonstrates that PCR amplification of genomic DNA sequences can be applied to crude DNA preparations from minute cells microdissected from archival smears. When the DNA segment to be amplified is restricted to 250 bp or less, most if not all archival smears can be used. This should meet the need for common molecular analyses based on PCR such as screening for gene mutation and LOH, and human papillomavirus typing. If it is necessary to target a slightly larger fragment, archival cervical smears could still be a valuable resource because at least a majority of cases can be used for PCR up to 460 bp. Overall, the quality of DNA samples prepared from archival cervical smears is better than

those prepared from archival formalin-fixed and paraffin-embedded tissues, which are often difficult to amplify fragments larger than 300 bp.[11,12]

After purification, DNA samples prepared from microdissected dyskaryotic cells that can be readily digested with restriction enzymes, such as *Hap*II, are therefore suitable for a PCR-based clonality analysis of the X-chromosome inactivation pattern. In keeping with the previous findings from cervical cancer biopsy tissues, our study based on archival cervical smears shows that mild dyskaryosis is polyclonal while severe dyskaryosis and a majority of moderate dyskaryosis are monoclonal. In addition to clonality analysis, such purified DNA samples prepared from archival smears should be adequate for methylation-specific PCR to study transcriptional suppression of tumour suppresser genes such as p16, which is implicated in the pathogenesis of cervical cancer.[13,14]

Based on microdissected dyskaryotic cells, we also demonstrated that archival cervical smears are suitable for RT-PCR. For a successful RT-PCR, a gene-specific primer, the antisense G6PD primer, rather than an oligo(dT) primer or random hexomers, should be used for cDNA synthesis. The oligo(dT) primer targets the 3′ end of RNA molecules and is not adequate for synthesis of cDNA from degradated RNA samples, such as those prepared from archival formalin-fixed and paraffin-embedded tissues and cervical smears. Random hexomers are a poor performer for reverse transcription with RNA samples prepared from archival fixed tissue/cells. The gene-specific primer targets the fragment to be amplified and is the best choice for RT-PCR of archival fixed tissue/cells. In addition, the fragment to be amplified by RT-PCR should be restricted to less than 150 bp. With increasing data on transcriptional profiles from microarray analysis, quantitative PCR to evaluate gene expression in a large series of cases is necessary. The RT-PCR protocol reported in the present study provides the basis to utilize such valuable archival materials.

In summary, archival cervical smears can be used for a variety of PCR-based molecular analyses at both DNA and RNA levels. In view of the huge resources of archival cervical smears and their long clinical follow-up, the potential to gain knowledge on cervical cancer by the molecular study of these archival materials is immense.

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