

Short report

Selective genetic analysis of p53 immunostain positive cells

M Phelps, B S Wilkins, D B Jones

Abstract

The isolation of p53 immunostain positive cells from histological sections for molecular genetic studies is a difficult task, especially if there are few positive cells. To eliminate contaminating DNA from p53 negative cells, which can obscure the results of molecular assays, a variation on the technique of immunohistoselective sequencing was developed. This is a highly selective approach, whereby immunostained sections of formalin fixed, paraffin wax embedded tissue are exposed to ultraviolet irradiation to damage the DNA in p53 negative cells. The DNA in positive cells remains unaffected because the dark immunostain protects their nuclei from ultraviolet light. Polymerase chain reaction single strand conformation polymorphism of samples enriched with p53 immunostain positive cells has shown that this method can produce pure samples of mutated DNA. The isolation of DNA from minority immunostain positive cells allows a wide range of molecular analyses to be carried out on these samples, which would otherwise be hampered by the problem of contaminating background cells.

(J Clin Pathol: Mol Pathol 2000;53:159-161)

Keywords: microdissection; polymerase chain reaction single strand conformation polymorphism; ultraviolet irradiation

The development of the polymerase chain reaction (PCR) has provided a valuable tool for the genetic analysis of individual populations of cells. One of the difficulties still associated with the investigation of small numbers of cells of interest is that of isolating the desired population from the surrounding normal stroma, DNA from which can obscure the results of molecular analyses.

Physical microdissection of areas of interest from histological sections has been used as a means of enriching tissue samples with the desired cells.^{1,2} When there are few target cells and they are dispersed within a tissue, microdissection can prove an impossible task.

Selective ultraviolet radiation fractionation³ has been used to isolate the desired cells, and can eliminate DNA from contaminating cells.

Target cells are protected by an ink dot and the whole section is exposed to short wave ultraviolet (UV) irradiation. The DNA in the unprotected cells is inactivated by UV light so that subsequent PCR only amplifies the intact DNA from protected cells.

Recently, an even more selective technique has been developed⁴ to isolate immunostain positive cells from a negative background. Immunohistoselective sequencing⁴ utilises the dark nuclear stain from p53 immunostain positive cells to protect against UV light; immunostain negative cells intermingled with the positive cells are inactivated. We describe a variation on the immunohistoselective sequencing method that successfully allows selective PCR amplification, producing amplicons purely from the protected DNA.

Methods

The tissue samples used were obtained from formalin fixed, paraffin wax embedded specimens in the pathology department archives. To validate the technique, five tissue blocks from four patients with carcinoma and known p53 mutations were analysed.

Shi and colleagues⁴ fixed fresh tissue in 80% ethanol before embedding in paraffin wax blocks. Routine fixation in formalin was the main factor responsible for our modification of their method. Fixation masks tissue antigens and an antigen retrieval step is required for successful immunohistochemistry.⁵ Glass slides were used instead of plastic film as support for tissue sections.⁴ Tissue sections were scraped off the glass slides for DNA extraction, rather than cut into small pieces as performed by Shi *et al.*⁴

The type of fixative used in tissue processing is known to affect PCR amplification.⁶ Because of the reduced amount of amplifiable DNA present in the sections a hot start nested PCR technique was used.

SECTIONING AND IMMUNOHISTOCHEMISTRY

Sections (6 µm thick) were cut and mounted on aminopropyltriethoxysilane (APES) coated glass slides. Different areas of the microtome blade were used for different blocks to avoid crosscontamination.

Tissue sections were dewaxed in xylene and rehydrated in 100% and 70% alcohol. Endogenous peroxidase activity was quenched with

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Accepted for publication
7 March 2000

0.5% hydrogen peroxide in methanol for 10 minutes, and antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) for 25 minutes in a microwave oven (800 W). Sections were then incubated with a monoclonal antibody against the p53 protein (1/500 dilution; Dako Ltd, Cambridge, UK) overnight at 4°C in a humidified chamber. After a 30 minute incubation with biotinylated sheep antimouse IgG (1/200 dilution; Amersham/Pharmacia, St Albans, UK) at room temperature, detection was performed using a peroxidase labelled streptavidin-biotin complex reagent (Dako Ltd). The peroxidase label was visualised by incubation with diaminobenzidine (DAB; Vector Laboratories, Peterborough, UK) for five minutes at room temperature. Twice the concentration of nickel solution recommended by the manufacturer was used in the DAB solution to increase the intensity of the immunostaining.⁴ After rinsing in Tris buffered saline (TBS) and washing in sterilised water, sections were incubated in 2.5% glycerol in water for three minutes to reduce the brittleness of the tissue¹ and allowed to air dry.

UV IRRADIATION AND DNA EXTRACTION

After immunostaining, the slides were placed in a UV light crosslinker (Model CL 100; Ultra-Violet Products Limited, Cambridge, UK) for varying lengths of time. The UV energy was set to 8000 µJ/cm² and the UV wavelength was 254 nm. UV exposure times needed for complete inactivation of DNA from p53 negative cells were determined for each exon by irradiating unstained sections for increasing lengths of time. For the cases analysed, the range of optimal UV exposure times for inactivation for each exon varied between 60 and 120 minutes (exon 5), 50 and 150 minutes (exon 6), 60 and 210 minutes (exon 7), and 30 and 120 minutes (exon 8).

After irradiation, 10–15 µl of digestion buffer (PCR buffer; 5 mM MgCl₂, 400 µg/ml proteinase K) was applied to each section, which was gently scraped off the slide with a clean sterile scalpel blade. The tissue was picked up with a sterile pipette tip and placed into a microcentrifuge tube containing 70 µl of digestion buffer. Two sections were placed in each tube, which was then incubated for 15–18 hours at

55°C. Proteinase K was inactivated at 94°C for eight minutes and tubes were centrifuged for five minutes at 10 000 ×g. A 5 µl aliquot of the supernatant was used as the substrate for PCR. For each run a negative control was included, to ensure complete inactivation of the background DNA.

NESTED PCR

PCR amplification of exons 5–8 of the p53 gene was carried out in 25 µl reactions containing 20 pmol of each primer, 200 µM of each dNTP, 1.5 mM MgCl₂, 0.75 units of HotStar Taq polymerase (Qiagen Ltd, Crawley, Sussex, UK), and the DNA template. Table 1 shows the primers used, which were derived from published sequences.^{4,7,8} For the first round of PCR, set 1 primers were used, and amplification consisted of an initial step at 95°C for 15 minutes for enzyme activation and DNA denaturation, followed by 35 cycles at 94°C for 30 seconds, annealing at the appropriate temperature (table 1) for 30 seconds, and extension at 72°C for 45 seconds. A final elongation step at 72°C for five minutes completed the PCR. For the second round PCR, set 2 primers were used and 1 µl of a 1/10 dilution of the product obtained from the first round PCR was used as the substrate, otherwise conditions were as above.

SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

Aliquots (5 µl) of the second round PCR products were mixed with 4 µl of loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol), denatured at 94°C for eight minutes, then quenched on ice before loading on to a mutation detection enhancement gel (FMC BioProducts, Rockland, Maine, USA).

Electrophoresis was performed in 0.6× TBE, at 120 V for five hours at room temperature using a water cooled system. PCR products amplified from normal tonsil DNA were used as negative controls. Bands were visualised using the PlusOne DNA silver staining kit (Pharmacia Biotech, St Albans, UK).

SEQUENCING

When sequencing was required, the relevant PCR reactions were repeated using seminested instead of nested PCR to ensure clear sequencing of the beginning of the exon. The primers used were the external F primer in conjunction with the internal R primer for each exon (table 1). Sequencing of PCR products was performed by Oswel Research Products Ltd (Southampton, UK). External F primers were used for the sequencing reactions.

Results and discussion

Specimens with known mutations were used to determine the length of UV irradiation necessary for complete inactivation of background DNA while retaining the ability to amplify the immunostain protected DNA. A series of sections containing p53 immunostain positive cells were irradiated for increasing lengths of time, the tissue processed, and SSCP analysis

Table 1 Primers used to amplify exons 5–8 of the p53 gene using nested PCR

Exon	Primers	Length of PCR product	Annealing temperature
<i>Set 1 (external primers)</i>			
5	F: 5' GTTTCCTTGGCTGCGGTGTTTC 3' R: 5' ACCCTGGGCAACCAGCCCTGT 3'	313 bp	64°C
6	F: 5' TGGTTGCCAGGGTCCCCAG 3' R: 5' GGAGGGCCACTGACAACCA 3'	223 bp	64°C
7	F: 5' CTTGCCACAGGTCTCCCCAA 3' R: 5' AGGGGTGAGCGGCAAGCAGA 3'	237 bp	64°C
8	F: 5' TTGGGAGTAGATGGAGCCT 3' R: 5' AGGCATAACTGCACCCTTGG 3'	281 bp	62°C
<i>Set 2 (internal primers)</i>			
5	F: 5' TTCCTCTTCTACAGTACTC 3' R: 5' GCCCCAGCTGCTCACCATCG 3'	214 bp	55°C
6	F: 5' GGCCTCTGATTCTCACTGATT 3' R: 5' AGAGACCCCACTTGCAAACC 3'	167 bp	57°C
7	F: 5' AGGCGCACTGGCCTCATCTT 3' R: 5' TGTGCAGGGTGGCAAGTGGC 3'	177 bp	58°C
8	F: 5' TGCTTCTCTTTTCTATCCTGA 3' R: 5' CGCTTCTTGTCTGCTTGTCT 3'	186 bp	60°C

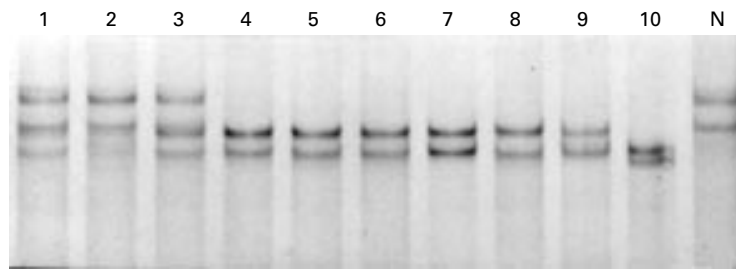


Figure 1 Single strand conformation polymorphism (SSCP) analysis of PCR amplification of exon 5 of the p53 gene after UV irradiation of tissue sections containing p53 immunostain positive cells. UV irradiation times were as follows: lane 1, no UV irradiation; lane 2, two minutes; lane 3, five minutes; lane 4, 10 minutes; lane 5, 20 minutes; lane 6, 30 minutes; lane 7, 40 minutes; lane 8, 50 minutes; lane 9, 60 minutes; and lane 10, 90 minutes of UV irradiation. N, normal control.

performed on the amplified products. Figure 1 illustrates the inactivation of normal DNA for exon 5. As the UV irradiation time increases, bands representing normal DNA disappear (lane 4), whereas the mutated DNA (identified by the shifted bands) can still be amplified. Direct sequencing of the shifted PCR product confirmed that it contained the same mutation as identified previously.

When unstained sections were used, UV exposure times for complete DNA inactivation were longer than the times established when protected cells were present. This could be because of preferential PCR amplification of the intact DNA over UV damaged DNA.

As for any method where low copy number targets are amplified, misincorporation by the DNA polymerase is a potential source of artefacts.⁹ A different mobility shift was detected in the sample in lane 10 (fig 1), and sequencing confirmed it to be the result of an alteration in the sequence, additional to the original mutation. When repeating the nested PCR for this sample, new products showed the original mobility shift rather than the altered one seen in lane 10. It is not possible to determine whether the introduced artefact was caused by the prolonged UV irradiation or was the result of misincorporation by the DNA polymerase during the first cycles of the first round PCR. In either case, the change in sequence would be introduced at a random place, and repeat experiments should confirm whether the identified mutation is genuine. A

random error is very unlikely to be introduced into the sequence at the same position every time. Careful optimisation and the use of hot start PCR¹⁰ are important for specificity and sensitivity in applications with low copy number targets.⁹ The modifications to the immunohistoselective sequencing method⁴ allow access to a wide range of archival biopsy material.

Selective amplification of a minority DNA population tends to pose a series of problems because the target DNA is surrounded by an excess of background molecules with which they compete during PCR amplification. By damaging the undesired DNA with UV irradiation and selectively amplifying the target DNA from p53 immunopositive cells we have obtained PCR products that contained only the mutated sequence, and have shown that this technique can be applied successfully to cases where the desired cells can be targeted by nuclear immunostaining.

The authors would like to thank Dr I Campbell for providing the sequencing information on the carcinoma cases used. This work was funded by the Association for International Cancer Research.

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Mol Path 2000 53: 159-161

doi: 10.1136/mp.53.3.159

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