Sensitive and reliable PCR and sequencing used to detect p53 point mutations in fine needle aspirates of the breast

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Abstract

Aims/background—Fine needle aspirates (FNAs) of breast lesions are now a routine investigation and prognostic information at this stage would be useful for accurate management. p53 gene status can be used as prognostic indicator, an abnormal genotype being associated with high grade, oestrogen receptor poor tumours. As the main disadvantage with FNA is poor cellularity, the objective of this study was to develop a sensitive and reliable method for the assessment of the p53 status of the lesion.

Methods—Using PCR and subsequent direct sequencing, a method was developed that enables analysis of the p53 gene from relatively few malignant or suspicious cells in a background of normal cells.

Results—This method is both reproducible and sensitive. The sensitivity of the method is demonstrated and a mutant cell can be seen in a background of 90% of normal wild type cells. A mutation, not previously described in breast cancer, is also reported in a symptomatic FNA.

Conclusions—This methodology is reliable and effective on samples with both variable cell numbers and quality of preservation, allowing it to be applied successfully to diagnostic cytology.

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Fine needle aspiration (FNA) is an established preoperative diagnostic procedure. As a sensitive method with high specificity, and because it is minimally invasive, it is readily acceptable to the patient as a diagnostic investigation. Additional advantages of FNA are the speed of processing and the relatively short time in which a report can be generated. The main disadvantage of FNA is the small numbers of cells made available for investigation, which illustrates the requirement for reliable and sensitive analytical methods.

FNAs of the breast are commonly used in both symptomatic patients and those referred for assessment from the National Breast Screening Programme. A diagnosis is made preoperatively and therefore appropriate patient management can be planned. In the case of benign breast disease, surgery may be avoided altogether.^{1 2}

The tumour suppressor gene p53 is the most commonly altered gene in human cancers. In breast cancer, a mutant p53 gene is present in between 20 and 30% of patients and this has been demonstrated to be of prognostic significance. A mutant p53 gene is also a valuable marker of malignancy and is often used as additional evidence for the presence of malignancy. The biological status of p53 can further be used to determine therapeutic response to either chemotherapy or radiotherapy.³⁻¹⁰

The knowledge of the p53 status of the patient at the preoperative stage of their management would enable the cytopathologist to give prognostic, diagnostic and possibly therapeutic information. This information may—for example, lead to decisions involving more radical surgery or adjuvant therapy, or both.¹¹

As DNA sequencing is the definitive method for determining the p53 status of the patient, we have developed a reliable and sensitive method involving PCR and direct sequencing to obtain this information from small numbers of cells obtained from FNAs of the breast.

Methods

CYTOLOGICAL COLLECTION AND PREPARATION

Specimens were collected from patients undergoing routine FNA of breast lesions. Aspiration was carried out at the time of imaging and conventional cytological smear preparations made. The needles used were subsequently washed out in RMPI 1640 (Sigma, Poole, Dorset, UK). It was possible to store these samples for up to five days at 4°C prior to preparation.

Needle washings were centrifuged in order to determine the size of the cellular deposit. The sample was then rediluted in an appropriate amount of RMPI 1640 prior to cytocentrifugation in order to achieve a monolayer of cells on the surface of a slide. One slide was immediately fixed in absolute alcohol for subsequent PAP staining. Other preparations were air-dried, wrapped in tin foil and stored at -70°C.

Cell numbers were estimated, unstained, by conventional light microscopy. Prior staining or fixation in alcohol/methanol was found to affect the quantity of PCR product gained adversely. The cells were then removed from the slide by rehydrating in an appropriate amount of sterile distilled water. Those samples with sparse cellularity (less than 50 cells) were diluted in 50 µl sterile distilled water.

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Table 1 Primers used in the sequencing reactions

Exon	Sequence	Strand
5	5'CCCCTGTCGTCTCTCCAGCC3'	Antisense
6	5'TAACCCTCCTCCCAG3'	Antisense
7	5'TCGGTAAGAGGTGGGC3'	Antisense
	5'AGGCGCACTGGCCTCATCTT3'	Sense
8	5'AGGGGGGTGGGAGTAGATGG3'	Sense

Those samples with greater cellularity were diluted accordingly—that is, those specimens where there was an even coverage of cells across the slides were diluted in 200 µl sterile distilled water. Negative controls consisted of sterile distilled water only.

Cell lysis was carried out as described by Kovach *et al*¹² with the following modifications. Rehydrated samples were diluted with an equal volume of PCR buffer (1X) which also contained 20 mM dithiothreitol, 1.7 mM sodium dodecyl sulphate and 0.1 mg/ml proteinase K. The digestion was carried out at 55°C for 60 minutes with subsequent inactivation at 95°C for 10 minutes. The samples were then centrifuged at 15 000 rpm for 10 minutes at 4°C to remove cell debris.

PCR AMPLIFICATION

The supernatant (10 µl) was used as substrate for nested PCR, which was comprised of two rounds using the following primers: first round PCR: 5' GCTCGCTAGTGGGTTGCAGGA 3' and (outer) 5' GCCCAACAACACCAGCTC-CTCT 3'; second round PCR: 5' CCGCT-TCTTGCTCCTGCTTGCT 3' and (inner) 5' TT TCTTTGCTGCCGTGTTCAA 3'.

Nested PCR produces a 1.6 kilobase fragment encompassing exons 5–8. Both sets of primers have identical optimal PCR conditions. The PCR mixture contained 0.5 µM of each primer, 20 µM of each dNTP, 1X PCR buffer, and two units of Taq polymerase (Boehringer Mannheim, Lewes, UK), made up to a final volume of 50 µl. The samples were placed on a Techne GeneE thermocycler (Cambridge, UK) for 30 cycles, comprised of 60 seconds denaturing at 94°C, 60 seconds annealing at 61°C, and an extension step at 72°C for 150

seconds. A lengthened denaturation and extension step preceded and followed the cycling part of the method; 5 µl of the product obtained from the first round PCR was used as substrate for the second round PCR.

Two reactions were carried out using a biotinylated form of either the sense or antisense primer to allow separation of the coding or non-coding strand with Streptavidin coated magnetic beads (Dynal UK Ltd, Wirral, UK). Strand separation was carried out as follows. Briefly, 160 µl of the second PCR product was purified and precipitated in 8 M ammonium acetate and isopropanol, allowed to dry and resuspended in 80 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Then, 80 µl Streptavidin coated beads was washed three times in Triton wash solution (TWS) (0.17% Triton X-100, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and resuspended in 80 µl TWS. The resuspended PCR product was diluted with a further 80 µl TWS prior to the addition of the beads. The mixture was placed on a shaker for 30 minutes. Denaturation of the strands was achieved by taking up the washed beads in 32 µl TE buffer and 8 µl 1 M NaOH containing 4 mM EDTA for two incubations of five minutes. The beaded PCR products were washed twice in TWS and taken up in 25 µl sterile, distilled water and stored at –20°C until sequencing reactions were carried out.

SEQUENCING

Sequencing was carried out according to manufacturer's instructions for the Sequenase version 2.0 Kit (Amersham International, Little Chalfont, UK). For each sequencing reaction, 7 µl of the PCR product/bead complex was annealed with 1 µl primer (1 µM) and 2 µl reaction buffer. Table 1 shows the sequencing primers used. The internal PCR primers were also used successfully as sequencing primers.

PCR SENSITIVITY DETERMINATION

The sensitivity of the PCR was established using control human tumour cells. These cells were harvested from imprints of tumour tissue in the same manner that FNA cells were prepared. The tumour cells had previously been fully characterised as diploid cells that possessed one wild type allele and one mutant allele. The mutation in these cells was a CGT to TGT base substitution at codon 273 (in exon 8). The mutation was seen, as expected, in association with a normal allele. Variable dilutions of the tumour cells against fully wild type cells (from normal tissue) was carried out to establish PCR sensitivity. The sequencing showed the presence of the mutation in all tracks.

Figure 1 demonstrates the unequivocal detection of the mutation when the genotypically altered cells are at a concentration of 10% in a background of normal cells. This therefore illustrates the mutant sequence in a background of 95% wild type sequence. A wild type sequence is also adjacent to these mutant

Figure 1 The ratio of mutant alleles to wild type alleles is: sample (1), 25%; sample (2), 10%; sample (3), 5%. Sample (4) illustrates wild type sequence.

Figure 2 Mutation at codon 275 (TGT to TTT) on the sense strand in the presence of the normal allele.

sequences in order to demonstrate and confirm the sensitivity and specificity of the PCR.

Results

The sensitivity experiments have demonstrated that one mutant allele can be detected in a background of 95% fully wild type alleles. This illustrates both the sensitivity and the extreme specificity that is characteristic of nested PCR.

Figure 2 shows the application of the methodology to cells obtained from a breast lesion in a symptomatic patient. The patient, aged 58 years at diagnosis, presented with a clinically and radiologically malignant mass. This mass was aspirated in clinic in order to obtain proof of the nature of the lesion, which was subsequently confirmed as malignant by cytology. The use of the PCR/sequencing methods described here demonstrated the presence of a mutation in the p53 gene. This mutation was detected at codon 275 (in exon 8) and is a known oncogenic change (a TGT to TTT substitution mutation with an amino acid change of Cys to Phe) (fig 2).

Discussion

Using the methods described, we have identified a mutation in a FNA sample. The patient's subsequent surgery (mastectomy and axillary lymph node clearance) revealed the presence of a 6 mm, grade III, invasive ductal carcinoma. Metastatic carcinoma was not detected in a total of 14 axillary lymph nodes. The molecular findings are in keeping with the known association of p53 mutation and high grade of tumour. Immunohistochemical staining of this excised tumour with an antibody directed against p53 (PAb 1801; Novocastra Laboratories, Newcastle, UK) showed overexpression of the protein, as would be expected by the detection of an oncogenic mutation which would stabilise the protein. The immunohistochemical method used involved a standard Streptavidin-biotin complex (ABC) method, with 30 minutes pretreatment in citrate buffer (pH 6.0) in a microwave oven.

Use of the Cariello p53 mutation database¹³ draws attention to the significance of the mutation reported here. The mutation has been documented previously in a non-small cell lung carcinoma,¹⁴ a B cell lymphoma¹⁵ and a colorectal carcinoma,¹⁶ but not in breast cancer. Other changes at this codon (a Trp and an Arg substitution) have been described in breast cancer.^{17 18}

PCR-SSCP is an alternative method used in many laboratories for the determination of p53 status. Preliminary work in this laboratory used additional PCR primers to generate fragments of 100–200 base pairs and compared SSCP results with sequencing results on samples derived from colon and liver tumours. This showed that the PCR/direct sequencing methodology described here has many advantages and SSCP was not therefore attempted on FNA samples.

The methods described are more sensitive; SSCP requires more of the sample to contain the mutation before a band with altered migration would be detected. Our methods are more reproducible; SSCP is highly dependant on small changes in electrophoresis conditions, permitting detection of each mutation. There are some mutations which will not produce bands of altered mobility and will never be detectable by SSCP. These techniques are unequivocal; the presence of a band with altered migration on an SSCP gel cannot be assumed to be a mutation rather than a neutral polymorphism or even a gel artefact unless it is sequenced or unless that particular mutation is run on the same gel as a control. Finally, the methods described here give more information than SSCP, allowing individual mutations to be correlated with outcome.

SSCP may have advantages when a very large number of samples need to be screened (most particularly when this is for one specific mutation of known significance) prior to sequencing, but cannot be recommended as a technique to be used alone to provide results for use in establishing prognosis, being prone to false positives and not capable of universal detection of mutations. We have taken the position that rather than using SSCP screening techniques on all breast FNA samples that arrive in the laboratory, a valid approach is to select samples for PCR/direct sequencing analysis from patients who will benefit most from knowing their p53 status. These fall into two categories: the first contains samples in which further diagnostic investigations are desirable—for example, when a definite diagnosis cannot be reached on the morphology alone. This group includes those samples classified as C3 (some suspicious or atypical features, but likely to be benign) and C4 (have features suspicious of malignancy, but not all criteria are met). The identification of a p53 mutation in these cases will enhance the confidence of a malignant diagnosis (although absence of a molecular abnormality will not resolve this predicament).

The second group of specimens that may benefit from molecular studies are those that require additional prognostic information.

FNA samples from patients who are being considered for neoadjuvant chemotherapy would fall into this group. Indeed, when studying p53 status in order to predict therapeutic response, molecular methods such as the PCR/direct sequencing described here are the methods of choice, so that any p53 mutations may be characterised fully. Null p53 status (due to frame shift or nonsense mutations)—for example, will lead to increased sensitivity to chemotherapeutic agents, whereas mutant p53 that has a gain of function mutation (mainly base substitution mutations) will have increased resistance to chemotherapy. Those patients with wild type p53 on both alleles can be predicted to respond to therapy, due to the involvement of p53 in triggering apoptosis.¹⁹ Precise data, such as these, cannot be determined by immunohistochemistry or PCR-SSCP alone. However, further work is required to fully evaluate the role of p53 in the chemotherapeutic response, as consistent data are seen rarely.^{20 21}

The main advantage of the methods reported is its extreme sensitivity in the presence of excess wild type sequences, and its reliability. There is a only a single variable in the method between samples, and that is the volume of diluent used to rehydrate the cells prior to proteinase K digestion. The method can also be completed within three to four days.

In summary, the methodology reported here is reliable and effective on samples with both variable cell numbers and quality of preservation, allowing it to be applied successfully to diagnostic cytology.

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